

Another taboo bites the dust

Squeamishness has too often in the recent past inhibited sensible inquiries into the mechanisms by which AIDS is spread through human populations but now there may be proof that these attitudes are changing.

THE survey of sexual behaviour in Britain described on pages 410–412 of this issue will be most vividly recalled as the survey Mrs Margaret (now Lady) Thatcher, when Prime Minister, refused to let government agencies back with public money (see *Nature* **341**, 181; 1989). In the event, the study (*Nature* **348**, 276–278; 1990) was made possible by a substantial grant from the Wellcome Trust, not then as flush with ready cash as it has since become. It is to be hoped that the outcome of the survey, together with the results of the comparable French survey described on pages 407–409 (which was supported fully from public funds), will at least show her that she was mistaken. Not that she is likely to say so publicly, for that is not her style.

But it would be wrong that the valuable data the two surveys have gathered should be remembered chiefly for the adventitious circumstances of the origin of one of them. AIDS was first recognized as a partly infectious disease (blood contamination also spreads it) almost exactly a decade ago, since when there has been general and understandable anxiety to understand the speed and the intensity with which an apparently novel epidemic would establish itself within exposed populations. From the outset (see Anderson, R. M. & May, R. M. *Nature* **333**, 514–519; 1988), it has been clear that data of two kinds would be necessary: data about the properties such as the incubation time of the infectious agent (then itself uncharacterized, but now known as human immunodeficiency virus, HIV), and data about the transmission routes, of which sexual transmission is the most important because it is the most obdurate. (Transfusion blood can and should be screened for contamination, for example.) A decade ago, little more was known of the frequency of various sexual practices among adult populations than had been learned about the United States population more than a quarter of a century earlier by the Kinseys.

Change

That state of affairs is now changing quickly, if at considerable cost. There have been surveys of the frequency of various sexual activities as functions of age, sex and social class, among other variables, in Norway, Denmark and even, the disapproval of the Bush White House notwithstanding, in the United States (Catania, J. A. *et al.* **258**, 1101–1106; 1992). The British and French surveys described in outline this week are more extensive than others, at least in the sense that the sample sizes, of the order of

20,000, are larger. There is, of course, ample room for argument among those who carry out social surveys of the technology that has been used. What, for example, are the relative merits of questionnaires administered by telephone or face-to-face? It is right and proper that specialists in the field should differ in their opinions on these points, but their conclusions are not invalidated by that circumstance.

Bias

One of the arguments quoted in exculpation of the Thatcher government's unwillingness to back the British study is that people questioned in any fashion about the most private details of their personal lives will lie, and will do so consistently, thus generating false confidence in mistaken conclusions. The premise is correct. People do lie when asked for intimate personal information, about salaries as well as about their private lives. Thus there is evidence of lying in this week's surveys, from each of which it may be inferred that men found more heterosexual partners than there were women acknowledging partnership. (The most likely explanation is that men are more boastful than women about sexual adventures, but the probable underrepresentation of female prostitutes in random samples may also be important.) There are two things to say about this familiar built-in bias. First, its magnitude seems to be declining with the passage of time, no doubt as the respondents to these studies are caught up by growing public awareness that sexual behaviour is natural. Second, and more practically, when random surveys such as these reveal a systematic bias, it is possible partly to correct for them by other kinds of studies. People's propensity to lie does not, in other words, make data of this kind useless, but merely qualifies the degree to which, without further study, they can be used for quantitative purposes.

So much is also plain from what the new surveys reveal about changing sexual behaviour. Indeed, it is remarkable how consistent are the new data, gathered by random survey, with the results of earlier studies and with anecdotal evidence. The steady decline over the years of the age of first sexual intercourse in France as well as Britain, and the similarity of the ages, is perhaps the most obvious proof of that. But the incidence of homosexual behaviour (underreported though it may be) is also remarkably similar. While there will be widespread and often prurient general interest in what these data show about the sexual behaviour of modern adult communities, there is no doubt



that they tell us more accurately than before what we and our fellows are like.

So what are the implications of the new data for the spread of AIDS? Will it now be possible to plug the numbers into a suitable mathematical model so as accurately to predict the future spread of AIDS in countries such as France and Britain? That expectation would be naive. As things are, uncertainties linked with the pathogenesis of AIDS, which may for example affect the definition of quantities such as 'incubation time', are probably as great as or even greater than those deriving from uncertainties of the behavioural data. In any case, what the world needs from a better description of the epidemiology of AIDS is not so much a precise estimate of its burden on health care services in decades ahead — the passage of time will sadly provide sufficient indicators of that — but an appreciation of the points at which changes in sexual behaviour may dramatically reduce the burden of AIDS on its potential victims and on society at large. In this connection, two features of this week's surveys obtrude: the relative promiscuity (compared with younger and older age groups) of people aged 35 to 45, and the evidence (from the French survey) that while there has been an encouraging spread of the use of condoms among the very young, large proportions of those most at risk continue to scorn the use of the only safe prophylactic against HIV infection so far known.

What will happen now? There is a good chance that the new surveys, like all good research, will be powerful stimulants of other investigations. It goes without saying that these data collectively will also help with the simpler problems of understanding the spread of other sexually transmitted diseases, too many of which are resurgent in modern society. There is the strongest possible reason why those who support research, governments in particular, should support continuing studies in this field and do so in a generous spirit. □

Waldegrave's dilemma

Mr William Waldegrave should not be deterred from consultation by his experience this week.

BRITISH science may not be what it used to be, but enthusiasm for reorganization remains apparently undimmed. So much is clear from the replies now trickling into the public domain from some of the many organizations invited to comment on the plan, early next year, to produce a white paper (policy statement) on research arrangements. Minor reorganizations happen all the time, of course, but if the promised white paper emerges, if it reflects in any way the radical ambitions of some of those who have commented upon it, and if it is then acted upon, British governments will have arranged for three major upheavals of public science in 30 years. But change — its pace and unpredictability — has become part of the problem, not the solution. British science would be better off if its institutions were this time given an opportunity to evolve constructively.

This time the pressure for radical change comes about for an unexpected reason — Mr William Waldegrave's decision to invite suggestions on the white paper project from organizations including the government's own bodies active in the field. Some of these have now published what they have had to say. The results are revealing, if more of the respondents than of their subject matter. The government's own Advisory Council on Science and Technology (ACOST), which argues for radical change, wants a "coordinated national framework for research" and a new research council "for scientific knowledge" called CASK, to support "curiosity-driven research".

There may be something in the idea, but the language in which it is embedded and the quality of the argument on which it rests leave much to be desired. Must researchers be known as "research providers", for example? Is it true that the "inordinate time . . . for peer review" is one of the objections to present arrangements for supporting basic research? Of course, if the "coordinated national framework" were sufficiently detailed, projects might be chosen by looking for appropriate keywords in the research grant applications. But would they necessarily be first-rate or even second-rate?

Waldegrave can easily slough off evidently pointless arguments such as these. He will find it more difficult, in the next few months, to keep at bay the clamour of those who would make the whole of the British research enterprise part of some yet to be defined industrial strategy. Yet that is the big danger; that having embarked on the laudable enterprise of looking again at the organization of research in Britain, Waldegrave will find himself lumbered with ideas whose only force is that they are advanced by powerful people. □

College admissions

Virginia education council says entering students should be able to read and calculate

It is increasingly the case that first year students at US colleges and universities require remedial courses in reading, writing and mathematics before they are fully able to take college-level courses. This absurd circumstance has occurred (in part) because of a political philosophy that says all students are entitled to a college education. Now, along comes the council of higher education for the State of Virginia with a remarkably sensible, if long overdue, suggestion for reducing the cost in dollars and time associated with teaching college students things they should have learned in high school.

Virginia's full-fledged colleges should raise their admissions standards, leaving students who are not prepared for college-level work to attend two-year community colleges where remedial courses more properly belong. It sounds so obvious. The sad part is that such a suggestion can be made seriously or deserve to be taken as novel. □

Gene therapy researcher under fire over controversial cancer trials

Washington. A scientific oversight panel at the US National Institutes of Health (NIH) has withheld funding for a contract supporting the work of Steven Rosenberg, an NIH cancer researcher, after criticizing Rosenberg for continuing to conduct controversial gene therapy trials despite evidence that crucial elements have failed to work as hoped.

Rosenberg has often been a controversial figure at NIH, both for his aggressive approach to cancer therapy and his tendency to get more press for the initiation of his trials than for their results. Now, for the first time, one of NIH's own advisory bodies has questioned the science and propriety of his trials.

In an unusually critical attack, the board of scientific counsellors of the National Cancer Institute's Division of Cancer Treatment has raised questions about several central aspects of Rosenberg's trials in which tumour-infiltrating lymphocyte (TIL) cells are extracted from cancer patients, grown in the laboratory, and transfected with the gene for tumour necrosis factor (TNF), a protein that interferes with a tumour's blood supply. The TNF-producing TIL cells are injected back into the patient, where they are expected to home in on tumours, infuse them with TNF and kill them.

But several important aspects of the TNF-TIL experiments have failed to work as hoped in the two years since Rosenberg

first gained approval to start his clinical trials with modified TIL cells. He has had continuing problems in getting the geneti-



Rosenberg: A history of aggressive trials

cally modified TIL cells to express TNF at levels high enough to affect the tumours. And, although his earlier experiments with unmodified TIL cells showed that they usually collect at tumours as expected, the TNF TILs do not appear to be performing

as well. Something about the insertion of the TNF gene into the TIL cells may be interfering with their homing ability. As a result, many appear to end up in the liver and elsewhere in the body, where the TNF could be toxic were it to be expressed.

At a meeting on 20 October, the board of scientific counsellors openly challenged Rosenberg, using the closest weapon at hand: a \$3.9 million, three-year contract to pay an independent laboratory to grow TIL cells. Rosenberg contends that the trials have shown enough success to be continued and have yielded significant insights that may lead to improved gene therapy approaches in the next generation of trials. Nevertheless, the board voted not to renew the contract until Rosenberg answered a list of questions on his progress.

Coming just as Rosenberg is preparing to launch a major new clinical trial using a new vector to put the TNF genes into TIL cells, the committee's decision to freeze funding for the TIL production could be a significant blow. Even if Rosenberg can satisfy the board, the contract will not come up for another vote until the board's next meeting in February.

Few of these concerns are new; indeed many of the same questions came up during the initial approval hearings by NIH's Recombinant DNA Advisory Panel (RAC) in July 1990. At the time, Rosenberg had little animal data to support his protocol when he presented it to the RAC; indeed,

Frustration brought panel to showdown

Washington. By all accounts, the showdown between Steven Rosenberg and the Division of Cancer Treatment board of scientific counsellors has been building for months as board members became increasingly concerned about what some felt were questionable trials with insufficient oversight.

When the board visited Rosenberg's laboratory in February, members raised a series of concerns about his gene therapy trial, including whether the genetically modified cells were expressing as much of a tumour-killing protein as projected and whether the cells were localizing at tumour sites. Yet at their 30 October meeting, they were no closer to getting the answers they wanted, and the frustration was starting to show.

"There was a lack of preclinical data for virtually every one of the issues that [Rosenberg] was propos[ing], and it would help if Steve provided the preclinical data to us. What we got instead were a series of preprints, none of which really answers those questions", complained Philip Greenberg, a University of Washington oncologist who is a member of the board. "His response to the site visit was not adequate."

The board has also become concerned about Rosenberg's

tendency to continue problematic trials until explicitly told to stop. Ralph Weichselbaum, a University of Chicago oncologist, noted that at the February 1992 site visit, "there were a series of — I don't want to be misquoted — not particularly successful trials [comparing high-dose interleukin-2 and interleukin-2 plus lymphokine-activated killer cells] that he was going to continue". The board recommended that he stop the trials, and Rosenberg eventually halted them in August. But Weichselbaum suggested that the experience indicated that some additional "constructive oversight" of Rosenberg's trials is in order.

Loretta Itri, another panel member, summed up the panel's frustration: "It seems to me that there have been straightforward questions asked. I am very disturbed that two meetings after the site visit we are still debating [them]."

As Greenberg put it, although the members generally support Rosenberg's work, "there [is] a difference between supporting the advance of science as opposed to supporting proceeding rapidly to clinical trials". Without clear evidence that Rosenberg's trials are working, the board apparently felt it had no choice but to assume the worst.

C.A.

even today, he says he knows of no researcher who has been able to reliably insert genes into primary mouse TIL cells (some other scientists say it has been done). Other data he presented, including proof that the TIL cells would express TNF in sufficient quantities, were simply sketchy.

In part due to the paucity of supporting data, several RAC members were concerned that Rosenberg's TNF-TIL trial appeared to blur the line between fundamental research and human clinical experimentation and questioned its description as gene 'therapy'. As Nelson Wivel, the executive secretary of the RAC, puts it, "the question is: do you call things therapy when they are so experimental that they don't have a good chance of success?"

In Rosenberg's defence, Bruce Chabner, the director of the Division of Cancer Treatment, argues that even if the TNF is not being expressed at sufficient levels, the patients may get some therapeutic benefit from the TIL cells themselves, which attack tumours on their own. But other scientists disagree; patients are eligible for TNF-TIL therapy only if they have already failed standard TIL therapy, they point out. More TIL cells that have already been proven not to work are unlikely to make a difference.

In part to answer its concerns, the RAC attached several restrictions to Rosenberg's protocol. One was that he could not inject the genetically modified cells unless they were expressing at least 100 picograms of TNF per ml (10^6 TIL cells) for 24 hours. But that level, it turns out, is at least an order of magnitude lower than necessary to shrink tumours. And in practice, Rosenberg has sometimes had trouble even reaching the RAC threshold. For the first TNF trial, he had to request special permission to average tests so as to get above the 100 picogram figure.

Even now, Rosenberg says, TNF expression in the trials varies from 100 to 1,200 picograms. Only a few patients show TNF expression at or above the 1,000 picogram level that he says the mouse models suggest is the minimum for efficacy. Rosenberg is hoping that the new vector may improve TNF expression by a factor of five. But he has as yet made no moves to halt the trials using the current TNF vector, despite the evidence that it has not worked as hoped — a point on which the board has challenged him.

Another concern of the board is whether Rosenberg's data shows that the gene-modified TIL cells are localizing to the tumours, and not ending up in the liver instead. TNF is extremely toxic and could, if expressed at sites other than the tumour, actually kill the patient before the cancer does. At the moment, the low level of TNF expression has avoided any toxicity problems, but the board has asked Rosenberg to supply new data to prove that the TNF is actually ending up at the tumour site. At the October meeting, Ronald Levy, chairman of the

board and a Stanford University oncologist, noted that Rosenberg's data so far showed that TNF-TIL localization is "about a quarter of a magnitude lower than was projected". Given that and the TNF expression problem, he said, "I just think that it is premature and unscientific to proceed with a clinical trial based on the pre-clinical or preliminary data that has been developed."

In general, the board is concerned that Rosenberg is trying in humans what he has not yet perfected in the test tube. TNF expression, for example, can be observed easily in the laboratory, yet Rosenberg embarked on trials before the expression problems were resolved.

Some researchers attribute the problems in the TNF-TIL trials to Rosenberg's unrelenting determination to be at the forefront of the competitive field of gene therapy and his belief that studies in terminally ill patients take precedence over fine points of *in vitro* research. In 1990 Rosenberg lost the race to be first in gene therapy to colleagues at NIH, who were treating a rare immune deficiency. But by then Rosenberg was clearly committed to going forward with his trial. In the two years since then he has inserted TNF-modified TIL cells into nine patients.

Rosenberg refuses to reveal the fate of those patients — most are believed to have died — until he publishes his results in a scientific journal. But his commitment to scientific standards has not kept him from publicizing, in everything from his new book, *The Transformed Cell*, to numerous press interviews, the fact that one of the patients is still alive. Whether the TNF experiment was actually responsible for that single remission is an open question.

One former collaborator (and now critic) believes that in the TNF TIL trials Rosenberg has "crossed the ethical line" in portraying research as therapy. Chabner concedes that in these trials "it's hard for us to say to a patient that you're getting a therapeutic level. The therapy issue is only part of the experiment. It may not work." But what you can prove with the trial, he says, is whether or not TNF gene transfer works in TIL cells. Noting that the patients in the study are terminally ill and have already failed other therapies, Chabner argues that the scientific value of the experiment is enough to justify the trials.

With the board of scientific counsellors in revolt, those arguments may no longer be enough. The board is expected to forward its list of questions to Rosenberg this week, and take up the issue again at its February meeting. Unless Rosenberg can provide them with better answers than he has over the past year, board members could make good on their threat to recommend that his TNF-TIL trials be halted.

Christopher Anderson

CERN blames full moon for LEP problems

Munich & London. Dogs howl, the tide rushes in and mild-mannered men turn into werewolves. Now scientists at CERN, the European Laboratory for Particle Physics in Geneva, have explained another lunar trick.

Mysterious variations in the energy of particle beams circulating in the Large Electron Positron collider (LEP) have been an unresolvable puzzle for years. The problem initially was not serious, but as experiments such as those to determine the mass of the Z^0 required increasing beam accuracy, a persistent uncertainty of 10 MeV became the dominant error in the LEP experiments.

Now CERN's particle physicists, along with colleagues from Stanford and Lausanne, finally have the answer. Tipped off by their



Driven round the bend: the LEP tunnel during the construction phase, before tidal effects had been considered.

California colleagues about the hazards of natural seismicity on beam energy, the LEP team realized that their variations were caused by minute deformations of the earth's crust due to the combined 'tidal' attraction of the sun and the moon. As the Moon passes overhead, it distorts the local radius of the Earth by 20 cm, changing the 27 km length of the accelerator by all of 1 mm. Because the number of revolutions per second of the particles in the beam is kept strictly constant, small changes in dimension oblige them to follow different orbits around the accelerator, which in turn either decreases or increases the beam energy.

The easiest cure, says Lyndon Evans of CERN, is to correct the data for the phases of the moon, although it could be possible to allow for tidal changes while experiments are running. That is easy enough; all LEP accelerator physicists have to remember now is to consult the phase of the moon — perhaps by checking the palms of their hands for hair — when calibrating beam energy.

Allison Abbott & Roland Pease

US genome project does it the French way, conceding that size matters after all

Washington. The US human genome programme is about to take a leap into big science, reassured by the knowledge that the French have been there first. This week, the US effort is expected to announce its biggest project so far — a \$24 million, five-year grant to set up a large genome mapping centre in Cambridge, Massachusetts — that in style and in scope will resemble nothing so much as the French Génethon, the industrial-scale gene factor near Paris that has revolutionized genome research in its first year of operations (see *Nature* 357, 526; 1992).

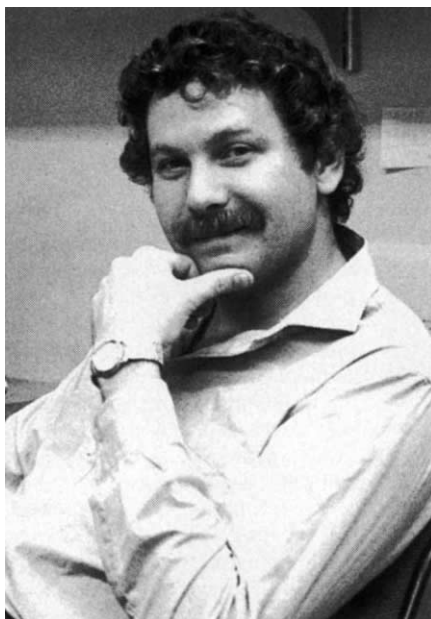
The likeness is not coincidental — Daniel Cohen, the director of the Génethon, is a collaborator in the project and will split his time between the centres. But the centres share philosophical roots as well. Like the Génethon, the Cambridge centre and a new \$13 million gene mapping centre to be based at the University of Iowa are focused on 'whole genome' mapping, rather than the chromosome-by-chromosome approach that has characterized the US effort so far. This represents a significant shift in the direction of the US project, something that, not surprisingly, has already met with resistance from researchers in smaller laboratories who view the transition to large centres as a threat.

The Cambridge centre will be headed by Eric Lander, a geneticist at the Massachusetts Institute of Technology's Whitehead Institute for Biomedical Research who already has a smaller centre for research on the mouse genome. With a team co-directed by David Page, another Whitehead geneticist who headed the group that constructed the first map of the human Y chromosome earlier this year, Lander will focus on obtaining a low-resolution physical map of the entire human genome, along with extending his mouse effort to obtain a high-resolution genetic map and a low-resolution physical map of the entire mouse genome.

Like the Génethon, the new US centres are taking a big-science approach to genome research. Lander's centre will combine nearly 40 researchers with robots and computer facilities to make up what will be the largest single fraction of the US effort. He has been collaborating with Cohen over the past year — the Cambridge centre will use libraries of clones developed at the Génethon — and the two scientists are setting up office space for each other in their respective facilities.

Only in their acceptance of industrial approaches to the genome do the two scientists differ markedly; Cohen, operating on private funds, has made the Génethon a gene

mapping factory, with robots operated mostly by technicians and an emphasis on throughput. Lander, on the other hand, is firmly in the academic model. His centre will be run mostly by postdoctoral researchers with their own research projects. In practice, this may mean that Lander's centre may not generate the sheer volume of markers of the Génethon, but its researchers may



Lander's centre is taking on whole-genome targets — and the critics.

spend more time doing genome analysis and technology development.

Lander hopes to put 10,000 sequence-tagged-site markers on the human genome, which would produce a physical map with 'anchors' an average of 300,000 base-pairs apart. For the mouse, he intends to obtain the same numbers of physical markers, including about 6,000 genetic markers.

The Iowa centre, based at the genetics laboratory of Jeffrey Murray, is the hub of a collaborative effort involving Iowa, the Marshfield Clinic in Marshfield, Wisconsin, Harvard University, and the Fox Chase cancer centre in Philadelphia, Pennsylvania. Together, the groups aim to obtain about 4,000 genetic markers to produce a high-resolution genetic map of the human genome.

Both larger than existing US centres and broader in scope, the two new centres have already stirred controversy. Some genome researchers — many of whom have grants due for renewal early next year — are concerned that the shift towards whole-genome mapping will leave little role for laborato-

ries focusing on fractions of the genome, such as single chromosomes.

There is, in fact, some reason for concern for such scientists. Certainly, the sort of basic genetic and physical mapping assignments that the centres have taken on may preempt similar work at smaller laboratories. One of the advantages of big, high-output centres, after all, is that they can achieve an efficiency of scale an order of magnitude above their bite-sized brethren. Lander expects that his centre will cut the cost of mapping a marker by at least a factor of four.

But that does not mean that the genome project no longer has a place for small science, Lander argues. His centre will produce only a relatively coarse map of the genome, containing only a third of the 30,000 marker target set by the genome project. Apart from the need to develop the remaining 20,000 markers, there is also much work to be done in exploring the functions and locations of the genes on the particular chromosomes. In those areas, smaller laboratories, in collaboration with dozens of other small groups, can be even more productive than large, central facilities.

A similar big-science versus little-science schism has already played out in the mouse genome community. Centres such as Lander's initially caused raised eyebrows, but when they demonstrated their willingness to share data freely long before publication, the fears that the smaller groups would be shut out of the race largely dissipated.

Indeed, Murray sees the existence of large centres as a new opportunity for some of the smaller teams in human genome research as well; not only will they now have access to vast amounts of new data, but they, too, can contemplate scaling up. His own laboratory, in fact, had been focused on chromosome four before he and his collaborators decided to join forces and propose a whole-genome approach.

Francis Collins, a University of Michigan geneticist who is under consideration as the next director of the National Center for Human Genome Research, calls the transition to large, whole-genome centres a "naturally evolving process" for the project, and notes that the timing is just as predicted in the effort's five-year plan. "A year ago everybody was wringing their hands and saying we'll never get there [to a whole-genome map]. Now they're saying we might be there a little too soon." If nothing else, he says, that is proof that it is coming right on time.

Christopher Anderson

Dutch win a reprieve over threatened lab closing

Munich. A decision to close one of the Netherlands most important research institutes, the Institute of Applied Radiobiology and Immunology (IARI), has been postponed following a government decision this week to look for another solution. The postponement, precipitated by a major outcry from Dutch scientists, gives hope that the institute and its world-renowned primate centre have a chance of reprieve.

Problems started when the recession-hit government asked the Netherlands' organi-

zation for applied research, the TNO, to generate more of its own income from industrial contracts; overall state funding for TNO's 25 institutes was reduced slightly in real terms this year, but much steeper cuts have been proposed for 1993. The TNO board of directors targeted ITRI for reorganization because it was running at a severe financial loss. But the scientific community has been quick to defend the institute, which specializes in diseases such as AIDS and malaria; concerted pressure has forced the government to look again at its plans.



The new Freddie Mercury AIDS centre, part of the threatened ITRI research institute.

zation for applied research, the TNO, to generate more of its own income from industrial contracts; overall state funding for TNO's 25 institutes was reduced slightly in real terms this year, but much steeper cuts have been proposed for 1993. The TNO board of directors targeted ITRI for reorganization because it was running at a severe financial loss. But the scientific community has been quick to defend the institute, which specializes in diseases such as AIDS and malaria; concerted pressure has forced the government to look again at its plans.

The forerunner of ITRI, the Radiobiological Institute, founded in 1956, was internationally renowned for its work under director Dirk van Bekkum on bone marrow transplantation, leading to the world's first successful treatment for aplastic anaemia. Van Bekkum went on to apply the research team's experience with nonhuman primates more broadly, and founded the Dutch Primate Centre in the early 1970s. The centre provided animals for wider studies at the research institute on infectious and autoimmune diseases.

But the running of two units was too expensive for the TNO management. In 1990, it decided to reunite the Radiobiological Institute and the Primate Centre, and over the next year 50 of the 220 staff were made redundant. But by 1992, the financial problems of ITRI were worse. In early summer

ITRI director Joost Haaijman was ordered by the TNO board of directors to offer a reorganization plan that would erase an estimated Dfl 3.7 million (US\$2 million) deficit. When Haaijman's plans were judged to be too moderate, he was replaced with a 'professional reorganizer', Jaap Geenen, whose more drastic plan involves closure of ITRI, with the loss of at least 70 staff, and merging of some of the research projects. Remaining staff would be transferred to the neighbouring TNO Medical Biological Laboratory. In addition, the Geenen plan suggests that the animal facilities be completely separated from research activities and transformed into an independent 'profit centre' for contract research. The plan was put forward two weeks ago to ITRI's 'employees' board', an elected body whose advice on changes in employment conditions must, according to Dutch law, be sought. The board has until the end of next week to put its case.

TNO at present sees no alternative to drastic action, given the government cutbacks in research funding. Health research in any case fits uneasily into TNO's portfolio of market-orientated technology centres; there is no market demand for much of the medical/biological research into the causes of diseases such as AIDS, malaria and multiple sclerosis, it says.

But Dutch researchers do not intend to let one of their leading research institutes go quietly. They are also dismayed at the speed of events — only months between the first announcement and the final decision — which leaves no time to look at realistic options such as European Communities funding.

The Dutch government has been taken by surprise by the force of the objections. More than 80 letters of support for ITRI have been received and questions have been asked in parliament. In response, Jo Ritzen, Minister for Education and Science, has given the researchers what they wanted — a breathing space. Ritzen is setting up an international committee, to be coordinated independently by the Dutch Royal Academy of Sciences, to reassess the international importance of ITRI and look for ways of saving it. But the governments has not said it will provide any more money itself.

TNO is now considering how it will handle the delay the government's action will force on its plans for ITRI and its primate centre. One TNO official asks: "Is the government prepared to pay for the centre in the meantime?" **Alison Abbott**

Biotechnology loses another battle in Germany

Munich. Hoechst, Germany's largest chemical company, has been blocked once again in its attempt to operate its human insulin plant. Permission granted in 1990 for the plant was last week revoked on a legal technicality — one more event in a long chain of delaying actions that have typified the eight-year battle to produce genetically engineered insulin in the German state of Hessen, which is dominated by the Green party.

Hoechst first applied for a licence for a human insulin plant in 1984, but five years of legal wrangling, with approval being alternately granted then revoked, led to a seemingly final decision against Hoechst in 1989 by the regional government of Hessen, one of Germany's 16 federal states. Hessen stated that there was "no sufficient statutory basis for the use of gene technology in Germany". During this time, other companies wanting to use such technology moved out of Germany to more politically sympathetic environments (see *Nature* 359, 93, 1992); but Hoechst had already invested too much in its project in Frankfurt. Finally, the advent of the German Gene Manipulation Act in 1990 provided the opportunity for a reversal of the decision, in what Hoechst believed to be the final stage of the battle.

But this was not the end of the story. According to the gene law, the biological system that Hoechst plans to use for engineering insulin is defined as posing "no risk for human health or the environment". This meant that heat inactivation of the waste water, a precondition of the 1990 approval, was no longer necessary. Hoechst put in a further proposal for a modified plant which excluded the inactivation step.

Approval for the modified plant was granted in July. But further objections were received by the Hessen environment ministry and the project was postponed once more until October. In the meantime, Hoechst appealed to Germany's administrative courts against Hessen's stalling tactics. But that backfired — the court instead ruled last week that the July authorization was invalid on detailed technical grounds — and Hoechst was back where it started.

Hessen must now decide whether to tidy up its authorization for the modified plant according to the directive issued by the administrative court, or to scrap it and start the process from scratch. It must also consider again the formal objections.

In the meantime, Hoechst claims to be losing DM3.5 million (US\$5.2 million) a month in lost income from the plant, and people suffering from diabetes are using imported genetically engineered human insulin from the United States and Denmark.

Alison Abbott

Japan topples tradition with move to foreign reviewers

Tokyo. In an unprecedented move likely to send shock waves through Japan's university research system, Tokyo University, the leading national university, has asked several eminent Western and Japanese scientists to carry out an external evaluation of its physics department, one of its strongest research-orientated programmes. The review,



Maverick: Arima

which will take place next month, will put pressure on university research departments throughout Japan to carry out similar reviews, because Japan's universities are now entering an era of increased competition due to a rapid demographic decline in

the student population that will continue over at least the next 20 years.

Most of Japan's universities are only just beginning to grapple with the concept of 'self-evaluation' thrust upon them last year by the University Council, an advisory body to the Ministry of Education, Science and Culture (Monbusho). But even self-evaluation is only an internal affair. Tokyo University's move takes evaluation two leaps further. Not only is the review external, like the regular reviews already carried out the Institute of Physical and Chemical Research (RIKEN, see *Nature* 359, 578; 1992) it will be the first external review of any research organization in Japan to involve non-Japanese scientists.

Under the self-evaluation system all of Japan's universities must produce annual reports showing their strengths and weaknesses. Next week, Tokyo University will release its report, or 'white paper' (*hakusho*) as it is officially called. This volume of more than 600 pages covers many problems, such as reform of the graduate school and the general education faculty, and points out deficiencies in personnel and facilities. But, as one of the deans of Tokyo University admits, the *hakusho* is largely "propaganda" and contains little "true evaluation".

The much more revolutionary external review of the physics department in January is the brainchild of Tokyo University's maverick president, Akito Arima, and the dean of the university's faculty of science, Ikuo Kushiro. At Arima's urging, Kushiro proposed the review to Arima, who came back with an "unexpectedly large budget", says Kushiro. That will enable the university to bring in several eminent scientists

from overseas, including Jean Audouze, science adviser to the French government, David Pines of the University of Illinois in the United States, Sydney Brenner, of the University of Cambridge in Britain, and Yoichiro Nambu of Chicago University.

There will also be several reviewers from Japan, among them Nobel prizewinner Leo Esaki, president of Tsukuba University, Minoru Oda, president of RIKEN, and Junjiro Kanamori, president of Osaka University.

Arima's choice of the physics department is not accidental — he used to work there and he knows that it is one of the best departments in the university. But, like all departments in Japanese national universities, the physics department is literally falling apart and suffers from a chronic shortage of staff, in particular technicians, due to a government policy that cuts the number of government employees (including staff of national universities) each year.

Arima, who has rushed the evaluation process forward before his retirement as president at the end of March, clearly hopes to use the external review as a shining example of what Tokyo University has achieved in the face of adversity. And he and Monbusho will undoubtedly employ the review to constrain the Ministry of Finance into giving the university more money.

Kimito Kubo, deputy director of the university division of Monbusho's Higher Education Bureau, says that the University Council had hoped to introduce such a process of external review, but had to settle for "self-evaluation" in the face of resistance from some universities. But such a review process is "absolutely necessary", he says, and it will have a "big effect" on Japan's other 97 national universities.

Tokyo University's head-start may even turn out to be canny strategy for more funding. There is a new mechanism by which Monbusho and universities can reward departments that come out favourably under such reviews. To improve graduate level education, the ministry this year introduced a new fund, the Special Fund for Promotion of Research in Higher Education, of ¥4,000 million (\$32 million). With the new fund, special grants of ¥10–100 million, over and above normal university funding, can be given to university departments or divisions carrying out exceptionally good research at the graduate level. This year the money was distributed fairly evenly, but next year Monbusho and the universities will probably begin to pick and choose between departments and research groups based on the evaluations now under way.

David Swinbanks

RITE more right for foreigners than Japanese

Kyoto. It seems Japan's young scientists are not yet ready to part with the tradition of lifetime employment. In an attempt to introduce Western employment practices to find the best talent and avoid lifetime commitments, the new, lavishly funded Research Institute of Innovative Technology for the Earth (RITE) this summer launched a recruitment drive offering high salaries — but nonpermanent positions — for the first jobs at RITE's main research facility which will open next year in Kansai science city between Kyoto, Nara and Osaka. But, while RITE has been inundated with applications from overseas, very few Japanese have applied.

RITE was established two years ago and has a budget this year of more than ¥6 billion (\$50 million). It arranges and funds joint research between government laboratories and industry to develop 'environment friendly' technology, such as biodegradable plastics and substitutes for chlorofluorocarbons.

With the opening of the new Kansai research facility next summer, RITE can begin its own in-house research. The new facility, destined to have a gleaming roof of solar cells that will provide about 10 per cent of the centre's electricity needs, is being built with donations of ¥4 billion from industry on a vast 40,000 square metre plot of land provided free of charge by Kyoto prefectural government, and it will have all the latest high-technology equipment. But a spacious environment, lavish facilities and the prospect of high salaries is proving insufficient to attract bright young Japanese.

RITE advertised extensively in Japan for applicants for the first 5–10 positions in the research facility, which will eventually have about 50 research personnel. RITE also placed one advertisement in *Nature* (*Nature* 16 July 1992, Classified 18). By the application deadline a few weeks ago, 63 people had applied. But, to the dismay of RITE, only 13 were Japanese; the remaining 50 were from 18 overseas countries, including the United States, France, the Netherlands, Russia, China, Sri Lanka, Hungary, Bangladesh, the Philippines, Pakistan, Iran, Bulgaria, Czechoslovakia and Mexico.

Japanese researchers appear to have been put off by the employment terms. RITE offers negotiable contracts of up to five years duration with salary dependent on qualifications. It is prepared to offer very generous salaries to the right people, but apparently Japanese shied away from applying because of the lack of guaranteed permanent employment, says Tsutomu Yamaguchi, RITE's senior managing director. In Japan, nearly all research jobs in government and industry guarantee lifetime employment.

David Swinbanks

More errors in errors

SIR — The Commentary by Menger and Haim (*Nature* 359, 666; 1992) calls for my response as editor of the *Journal of the American Chemical Society* (JACS). Menger and Haim complain about the peer-review process and the “considerable anguish” they suffered during consideration of their manuscripts by JACS. I am sure many authors could write more interesting and valid discussions about the problems they have had getting their work published. Most scientists, however, are sufficiently mature and amicable that they do not feel it necessary to document for all to see their tribulations in their interactions with their peers and editors.

Since the Commentary dealt with correction of errors, it is appropriate to point out some of Menger and Haim's own errors. For example, they state that “events began with the publication of two Articles”, while in fact the main paper in question was a “Communication”. This is more than a technical point. JACS makes a clear distinction between Articles and Communications. In our Notice to Authors (*J. Am. Chem. Soc.* 113, 10A; 1991), Communications are defined as reports (usually preliminary) that are limited in length to about 1,000 words. This Notice to Authors, incidentally, also states that “Notes” and “Comments” are not published in JACS. Thus, Menger's and Haim's papers submitted to JACS were largely commenting, without the presentation of any new results, ideas or concepts, on a brief communication limited by its size to the amount of experimental detail or discussion that it could contain.

It is JACS policy that we do not publish minor corrections as separate publications that do not contain new results. (Authors can, however, publish corrections in a separate Additions and Corrections section.) JACS has a fixed page budget which is heavily oversubscribed. If a three-page correction is published, then two communications containing original new ideas will not be. Corrections should be published if the point made is important. However, in the view of the reviewers and the editor, Menger's and Haim's work fell in the category of minor corrections and did not seem to affect the basic ideas of the Breslow *et al.* work. I ultimately decided to publish Haim's manuscript, after considerable revision based on valuable comments by reviewers and the editor, so it is difficult to see why he is complaining.

No pressure was brought to bear on the JACS editors not to publish the Menger and Haim manuscripts. Indeed, the only attempt to influence the edito-

rial process outside the usual channels was made by Menger, when he contacted the ACS publications committee and the ACS president. The ACS rightly maintains a hands-off approach in the management of its journals; so these attempts had no effect.

Finally, one can question the propriety of publishing (sometimes out of context) reviewer's comments and the contents of private correspondence. Overall, I am afraid the Commentary says more about its authors and the journal that agreed to publish it than it does about the peer-review process.

Allen J. Bard

(Editor, *Journal of the American Chemical Society*)
Department of Chemistry,
University of Texas,
Austin, Texas 78712, USA

Freedom to explore

SIR — I agree with the author of the recent leading article “High time for the circuses to stop”, who points out that we will continue to be enriched by increasing our knowledge of the Solar System (*Nature* 358, 609; 1992). However, I disagree with his assessment of the tools needed in this endeavour. Space Station Freedom will be a pivotal tool — one in a progression of tools dedicated towards this goal.

A common complaint among the uninformed is that Space Station Freedom won't do science. I ask the readers of *Nature* to look at the building where their laboratories are located. Does the building itself do science? No — it is how the building is fitted with equipment and how that equipment exchanges data with other researchers that makes it possible for research to be performed. Space Station Freedom is designed to be a research facility in space capable of being reconfigured over its 30-year lifetime to meet an ever-changing mix of basic and applied research.

By visiting space we are exploring a new frontier. The first explorers of terrestrial continents brought back not only gold and commercial materials, but many new plant and animal specimens. As exploration progressed, the capabilities of exploration vessels advanced. The overtly exploratory travels of Marco Polo, Charles Darwin, and Lewis and Clark, and the considerable scientific data produced as the direct result of their expeditions, show what can happen when curious people are sent to examine the frontier.

A gravitational field of 1G is the single environmental factor to have remained

unchanged throughout life's tenure on this planet. To perform long-term research in the absence of gravity, one must go into space with a permanent laboratory. Chronic removal of that evolutionary constant will most certainly provide new insight into the ontogeny and phylogeny of life on Earth.

The lack of gravity also has a profound effect upon many physical processes. Convection, buoyancy and thermal stratification do not occur. In addition, comparatively weak physical interactions normally masked by stronger phenomena often prevail in microgravity. We have only begun to understand the effects of this novel environment on metallurgical, chemical and combustion processes. Some of the more promising results are in organic and inorganic crystallography. It is impossible to predict what new technologies and new insights will come from space research. But as new knowledge is gathered, they will surely come.

Finally, *Nature's* leading article suggests that Space Station Freedom might become a circus. To this challenge I respond “long live the circus”. A circus, by dictionary definition, is a “pageant featuring acts of skill and daring”. Such is the exploration of space.

Robert W. Phillips

Chief Scientist,
Space Station Freedom,
National Aeronautics
and Space Administration,
Washington, DC 20546, USA

Photofat

SIR — We feel it necessary to comment on Daedalus's column (*Nature* 359, 20; 1992) concerning breast enlargement for women. Does Daedalus believe that such new and improved women will have anything to do with average men? Ha! Such women will require new and improved men as well. Unfortunately, Photofat will not work, as the parts of men's bodies in need of enlargement contain few fat cells. We're sure Daedalus will agree with the necessity of a comparable enlargement scheme for men, considering the puniness of the average man's muscles and other parts. We assume he will not object to the frivolous alteration of a healthy, normal male body, since he has no trouble imagining this for women.

Susan M. Purcell

Virgnetta S. Cannon

Carmen R. Domingo

S. B. Minsuk

Department of Molecular
and Cell Biology,
315 Life Sciences Addition,
University of California,
Berkeley, California 94720, USA

Plight of Wei Jingsheng

SIR — We wish once again to direct the attention of the world scientific community to the dire plight of Wei Jingsheng, a political prisoner in the People's Republic of China. Imprisoned 13 years ago (on 16 October 1979) for pro-democratic writings, Wei is reported by confidential sources to be in poor health under harsh conditions, and possibly in danger of dying before completing his 15-year sentence.

As oceanographers, our concern was aroused by Chinese pamphlets calling for Wei's release found in a bottle by one of us (R.S.) in June 1990, on the west coast of Vancouver Island, British Columbia, Canada. This finding, once communicated to Beijing, provides Deng Xiaoping with an honourable justification for pardoning Wei Jingsheng before his sentence — and his health — expires.

After analysis (described in a manuscript submitted to *Eos* for publication), we conclude that the bottle was one of many released by Taiwanese activists shortly after Wei's imprisonment. In itself, the bottle is scientifically unremarkable. The inferred course of the bottle from its probable release off the islands of Quemoy or Matsu close to the Chinese mainland is consistent with known ocean currents as verified by computer simulation. Probably 50 per cent of the bottles reached their intended target of mainland China, and bottles containing similar literature have been reported from coastal Japan.

The significance of the finding lies in the fact that the bottle was found by an oceanographer and transmitted to a colleague (C.C.E.) studying drifting objects. We estimate that fewer than one per cent of the bottles released would have reached the sparsely populated northern coast of North America more than 10,000 km away. Most such notes, even when found, are never translated, let alone interpreted.

We call Deng's attention to a passage in the Japanese legend, The Tale of the Heike, that he could cite as a precedent for releasing Wei. In the year 1177, the priest Yasuyori was exiled for political subversion to an island 100 km offshore. From his exile, he released 1,000 *stupas* (thin wooden boards offered as graveside memorials), each bearing his name, the date and two poems. Against steep odds, one *stupa* was found by a priest on the mainland. He transmitted it to Yasuyori's family and thence to the emperor and to Kiyomori, head of the Heike, who had ordered Yasuyori's exile. Two years later, upon the birth of a grandson and heir, Kiyomori pardoned Yasuyori.

We recognize the formidable obstacles

to obtaining Wei's release. His fellow scientist Fang Lizhi petitioned Deng on Wei's behalf in a letter in January 1989. Influential organizations such as Asia Watch, Committee to End the Chinese Gulag and Amnesty International have been working hard for the freedom of Wei and numerous other Chinese prisoners for many years.

Nevertheless, we hope that Deng will view this rare finding and deciphering of a message in a bottle by scientific 'priests' — and our communication of it to the latter-day 'emperor' in keeping with the 800-year-old Asian legend — as an omen and an opportunity to pardon and possibly spare the life of Wei Jingsheng.

Curtis C. Ebbesmeyer (*Evans-Hamilton, Inc., 731 N. Northlake Way, Suite 201, Seattle, Washington 98103, USA*); **W. James Ingraham Jr** (*National Oceanic and Atmospheric Administration, Seattle, Washington 98115, USA*); **Richard McKinnon** (*Asian Languages and Literature, University of Washington, Seattle, Washington 98195, USA*); **Akira Okubo** (*Marine Science Research Center, State University of New York, Stony Brook, New York 11794, USA*); **Richard Strickland** (*Washington Sea Grant Program, University of Washington, Seattle, Washington 98105, USA*); **Dong-Ping Wang** (*Marine Science Research Center, State University of New York, Stony Brook, New York 11794, USA*); **Peter Willing** (*Water Resources Consulting, Bellingham, Washington 98226, USA*)

Women in science

SIR — We are pleased that, in your comment¹ on the practice of the National Science Foundation to require evidence of the participation of women in scientific conferences supported by public funds, you recognize that the practice is laudable, but you underestimate the magnitude and subtlety of the challenges faced by women in science, especially in relation to meetings.

Although the numbers of women studying and teaching science in colleges and universities are increasing, they are still under-represented in the higher academic ranks of tenured positions². Part of the explanation is that to gain tenure one must demonstrate the respect of one's colleagues, which is in part measured by activity at meetings, especially by invited participation.

In scientific communities, as elsewhere, informal networks of colleagues effectively preside over their respective fields. When sponsoring or proposing a meeting, these networks strongly influ-

ence who is invited to contribute and often identify appropriate speakers from their ranks. Thus such networks tend to function, often unwittingly, as closed units, connecting people who share an intellectual pedigree and giving to meetings a restricted view of the potential intellectual breadth of a discipline. More subtly, because such restricted meetings may serve to galvanize a field, they further narrow the field's mindset, and strengthen the network that created the meeting.

Entering such a network from the outside is difficult for both men and women, but one important mechanism for circumventing this and other problems is through mentors³, established professionals in the field who advocate the merits of an outsider and his or her ability to further a scientific field. The problem lies in developing a mentor relationship. This requires personal contact with professionals in the field, which is most easily established by active participation at meetings. Unless the circle is broken, the representation of women in science will not increase.

Increasing the participation of women in scientific conferences is one way to facilitate the entry of women into the controlling networks, to help their research programs excel and to increase their entry into tenured positions.

J. Engel
J. J. Flynn
P. R. Crane
S. Lanyon
Center for Evolutionary and Environmental Biology, Field Museum of Natural History, Chicago, Illinois 60605, USA

1. *Nature* **359**, 92 (1992).
2. Benditt, J. (ed.) *Science* **255**, 1363–1388 (1992).
3. Gibbons, A. *Science* **255**, 1368 (1992).

Wrong book

SIR — There is nothing like reading the original sources. In the Opinion piece discussing the Vatican's grudging resurrection of Galileo (*Nature* **360**, 2; 1992), you presume that the Catholic Church's stand was based on "the account of the Creation in Genesis". There is in fact no mention in that text of the motion of the Earth or the Sun. The reference you want is from Joshua 10:12, "Sun stand thou still". The Church's argument was that these words would not have been spoken had the Sun not been moving.

Robert J. Wyman
Yale University, Department of Biology, Kline Biology Tower, PO Box 6666, New Haven, Connecticut 06511–8112, USA

Efficacy of leprosy vaccine

SIR — C. L. Crawford¹ begins by criticizing a statement in *Nature* by K. S. Jayaraman² that the report by Convit *et al.* in the *Lancet* earlier this year³ was the first demonstrated effect of BCG on leprosy in the New World because "this conclusion is drawn from a retrospective case-control study, which is different from the randomized double-blind trial...". Jayaraman's statement is correct. It is also true that the observation by Convit *et al.* was based on a case control analysis, but this does not invalidate their conclusions. Case control methods are now a well established method of estimating vaccine efficacy^{4,5}, and thus the issue is whether the Venezuela study was properly conducted — as we believe it was. Beyond this, more than 15 published studies, including cohort and case control studies, as well as randomized trials, have examined the effect of BCG on leprosy, and all have found significant protection^{6,7}. Available evidence indicates that BCG vaccines are in fact more effective against leprosy than against tuberculosis^{6,8}.

Crawford then expresses "surprise" at the omission of a placebo arm from the continuing leprosy vaccine trials in Venezuela and Malawi. The reasons are well known, and have been published^{3,9}. In the Malawi study, the protective effect of BCG was established before the start of the trial¹⁰ and it was therefore judged unethical to include a placebo arm. In the Venezuela study, the protection conferred by BCG had not been established at the start of the trial, but the number of leprosy cases expected in the population was judged too small for a three-arm comparison. In evaluating a new vaccine, the question of public health importance is not the absolute efficacy of the new preparation but whether it can improve upon vaccines already being given to the population.

The points in the final paragraph of Crawford's letter are unclear to us, but may imply that declines in the incidence of leprosy and in particular of multibacillary disease, in some trial populations, have been a consequence of case finding and chemotherapy. There is no clear evidence for this, and, in any case, it is irrelevant to the issue of vaccine evaluation in a randomized trial. Declines in leprosy have been recorded in many populations in the absence of chemotherapy, and are expected as a consequence of improved socio-economic conditions¹¹. Available evidence indicates that the protection imparted by BCG against multibacillary disease is as great as that against paucibacillary disease^{7,8}. This is consistent in all published studies with the exception of the

Uganda trial (which included only a single multibacillary case)¹² and one small study described only in a letter¹³. We believe that there is now overwhelming evidence of the effectiveness and impact of BCG vaccination against leprosy. BCG has been delivered to populations mainly as a vaccine against tuberculosis, and over the past three decades some 3 billion doses have been administered globally. This is certainly one of the factors responsible for the decline in the incidence of leprosy observed in many countries in recent years⁷.

P. E. M. Fine

P. G. Smith

*Department of Epidemiology
and Population Sciences,
London School of Hygiene
and Tropical Medicine,
Keppel Street,
London WC1E 7HT, UK*

1. Crawford, C. L. *Nature* **359**, 100 (1992).
2. Jayaraman, K. S. *Nature* **356**, 373 (1992).
3. Convit, J. *et al. Lancet* **339**, 446–450 (1992).
4. Smith, P. G. *Tubercle* **62**, 23–35 (1982).
5. Orenstein, W. A., Bernier R. H., & Hinman A. R. *Epid. Rev.* **10**, 212–241 (1988).
6. Fine, P. E. M. & Rodrigues, L. C. *Lancet* **i**, 1016–1020 (1990).
7. Fine, P. E. M. *Int. J. Leprosy* **60**, 71–80 (1992).
8. Ponnighaus, J. M. *Lancet* **339**, 636–639 (1992).
9. Fine, P. E. M. & Ponnighaus J. M. *Trans. R. Soc. trop. Med. Hyg.* **82**, 810–817 (1988).
10. Fine, P. E. M. *et al. Lancet* **ii**, 499–502 (1986).
11. Fine P. E. M. *Epid. Rev.* **4**, 161–188 (1982).
12. Stanley, S. J. *J. Hyg. (Camb)* **87**, 233–240 (1981).
13. Abel L. *et al. Lancet* **i**, 1536 (1990).

Travel restrictions

SIR — During the Cold War, the communist regimes of East Europe sought to restrict freedom of travel for all their citizens, even those whom they allowed to travel abroad for science or business reasons. Thus in Poland scientists and businessmen were issued with special passes called service passes. The communist government urged Western nations not to issue visas unless a visa application was supported by a letter from a Polish authority (for example an embassy). The aim was to prevent Polish nationals from changing their route while already in the West. Most nations refused to treat such passes differently from normal Polish passes, but Spain, France and Italy signed consular agreements that they would request such letter before considering visa applications. As such a visa granted no extra privileges, its discriminatory nature was clear. Thus a Polish researcher elsewhere in Western Europe could pay a short visit to a French colleague only if he was behaving well and was prepared to wait three months or more for a permit from the Polish Embassy. The practice had nothing to do with the

national interests of these Western nations since they surely would not be protected by Polish communists. On the contrary, I heard of scientists being blackmailed by the Polish authorities issuing such letters to collaborate in gathering intelligence against the collaborating Western nations.

The Cold War is over, the communist regimes vanished, Polish KGB-men charged with issuing such visa permits in Polish embassies are all unemployed, but Spanish diplomats still request their letters before issuing visas. The practice was always immoral. Now it is also anachronistic.

Alex Schwarzenberg-Czerny

*European Southern Observatory,
Karl-Schwarzschild-Strasse 2,
D-8046 Garching bei München,
Germany*

British science

SIR — Terence Kealey bemoans the imbalance in the origins of scientific effort between industrialized and developing nations¹. In his desire for equality, his response in endorsing a slower growth of British science to redress the imbalance is injurious to the very people he seeks to help². Beneficiaries are not limited to the geographical site of publication of the science. In the global village, both disease and scientific advances are quickly shared.

For example, the emergence of multi-drug-resistant strains responsible for pneumonia, malaria and tuberculosis (the largest cause of death from a single infectious disease) is a worldwide concern³. In applying the techniques of molecular genetics to target the pathogens causing tuberculosis, the recent work of Zhang *et al.* demonstrates its applicability to both North and South, despite the Anglo-French origin of the authors' collaborative efforts⁴.

This specific example is illustrative of the general proposition that, as in all trade, knowledge transfer is beneficial to both participants in the transaction. Knowledge transfer is less costly than autonomous knowledge development and frees resources, promoting long-term growth prospects and life quality in the recipient nation.

It would be cruel if, in the name of equality, a slower growth of science were somehow welcomed. That would truly be inequitable to potential beneficiaries, North and South.

Edward Krowitz

*2415 North Dickerson Street,
Arlington, Virginia 22207, USA*

1. *Nature* **358**, 533 (1992).
2. *Nature* **357**, 272 (1992).
3. Bloom, B. *Nature* **358**, 538 (1992).
4. Zhang, Y. *et al. Nature* **358**, 591–593 (1992).

AIDS and sexual behaviour in France

ACSF investigators*

The results of a massive telephone survey of sexual lifestyles in France should provide a basis for prevention strategies for AIDS and sexually transmitted diseases.

WITH sexual transmission of the human immunodeficiency virus (HIV) now known to be widespread in northern Europe and North America¹, many countries in these areas have decided to undertake surveys of sexual behaviour in the general population. The aim of these studies is to achieve better-defined strategies for preventing sexually transmitted diseases (STDs) and AIDS, as well as to provide the basis for more accurate models to determine the development of the epidemic². Some preliminary studies have already been conducted in countries such as Norway³, Denmark⁴ and Scotland⁵. Although the study of sexual behaviour has been amply legitimized by the context of AIDS, this legitimacy cannot unfortunately be taken for granted everywhere^{6,7}.

In France, a survey on sexual behaviour in a representative sample of the general population was carried out in 1970 arising from the increasing use of medical contraception⁸. In response to the spread of the AIDS epidemic, the French government asked the *Agence Nationale de Recherches sur le Sida* (ANRS) to perform a survey of sexual behaviour and AIDS in the general population (called ACSF for *Analyse des Comportements Sexuels en France*). To our knowledge, this study is the most comprehensive of its kind. It has been constructed in a way that can provide a

basis for comparisons with other countries, particularly with the British study, which deals with a similar sample in a comparable country⁷.

Our work began in July 1989. Three pilot surveys were carried out in the first 2 years to test the questionnaire, decide on the method of investigation (telephone or face-to-face) and to see if sending out a notifying letter affected whether people would participate in the study. The results prompted us to choose the telephone method for the national survey, with a notifying letter being sent beforehand^{9,10}. The population base was assembled from a list of all the telephone numbers in France (94% of French households have a telephone). A total of 110 interviewers took part in the survey from September 1991 to February 1992, following a 2-day training period. Each telephone number was tried for up to 12 times.

The first part of the questionnaire, which lasted 15 minutes, was designed to measure the prevalence of a number of risk indicators: homosexual/bisexual intercourse; intercourse with prostitutes over the past 5 years; multiple partners; and drug consumption in the past year. This part of the survey was applied to everyone questioned: a total of 20,055 people aged 18–69 years. An additional questionnaire lasting around 30 minutes was administered to all people replying to the first questionnaire who reported one of the above risk indicators ($n = 2,271$), and to a control group of people selected at random by their date of birth ($n = 2,549$)¹¹. The longer questionnaire included a sexual biography and details of several psychological and social characteristics.

Acceptability and reliability

Considerable efforts were made to maximize the response rate of the survey and to obtain the most reliable information possible. To convince the interviewees that their answers would remain anonymous, a letter explaining this policy was sent to all the selected households beforehand. All identifying information (name, address, telephone number) was automatically destroyed as soon as the reply to the first question was entered into the computer system.

Once selected, a household was informed that the aim of the survey was to make prevention of disease more effective,

and that the survey was being conducted by researchers employed by public-health institutions. The theme of AIDS and sexual behaviour was deliberately not mentioned in the letter to avoid worrying people, to prevent refusals before selection of the interviewee and to prevent people from preparing answers in advance. (The results of our pilot survey indicated that this strategy would be sensible.) Once the eligible person had been randomly selected from within the household, he or she was informed that the questionnaire concerned AIDS prevention and included questions on sexual behaviour. Given that AIDS can be sexually transmitted, the questions referring directly to the disease were placed after questions on sexual behaviour to minimize the possibility of people inaccurately claiming 'socially desirable' behaviour, such as condom use.

A random sample was used, first to ensure compatibility with surveys in other countries, and second because quota sampling leaves a broad freedom of choice in which subjects are interviewed, and generally leads to higher reported proportions of multipartner or homosexual individuals¹². To minimize recall problems, it was decided to focus on a detailed description of sexual practices and means of protection used in the last two sexual encounters that may have taken place in the year preceding the study¹³. This policy is identical to that adopted in the Norwegian survey³. A linguistic analysis of 100 telephone interviews during the pilot study allowed us to adjust the wording of the questionnaire so that the language used would be as clear and neutral as possible, without using words that were considered either too technical or over-vernacular¹⁴.

A total of 40,000 telephone numbers were drawn at random from all the telephone numbers in France, of which 30,157 were potentially eligible. The refusal rate for the households contacted was 11.8%. Subsequently, 11.6% of selected individuals refused to participate. Only 7% of these refusals were prompted by a reluctance to answer questions on sexual behaviour.

If households where no one could be contacted are not taken into account (that is, if we assume that these people would have refused at the same rate as those who were contacted), the refusal rate was 23.5%, very close to the rate

* ACSF (*Analyse des Comportements Sexuels en France*) investigators: Alfred Spira (Director), Nathalie Bajos (Coordinator), André Béjin, Nathalie Beltzer, Michel Bozon, Béatrice Ducot, André Durandau, Alexis Ferrand, Alain Giami, Augustin Gilloire, Michel Giraud, Henri Leridon, Antoine Messiah, Dominique Ludwig, Jean-Paul Moatti, Lise Mounnier, Hélène Olomucki, Jeanine Poplavsky, Benoit Riandey, Brenda Spencer, Jean-Marie Sztalryd, Hubert Touzard. ACSF is a survey from the *Agence Nationale de Recherches sur le Sida* (ANRS). It is conducted and coordinated under the scientific responsibility of INSERM Unit 292, and the survey associates researchers from INSERM, INED, CNRS and universities Paris V, Paris VII, Paris XI and Paris XIII. This study is supported by ANRS, the *Direction Générale de la Santé* (DGS), the *Comité Français d'Education pour la Santé* (CFES) and the *Agence Française de Lutte contre le Sida* (AFLS — Agency for AIDS Prevention). Correspondence should be addressed to A. Spira at Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France.

TABLE 1 Results of 20,055 questionnaires (including 4,820 long questionnaires)

	MEN				WOMEN			
	Age (years)				Age (years)			
	18-24	25-34	35-44	45-69	18-24	25-34	35-44	45-69
Population	1,716	2,232	2,284	3,696	1,670	2,238	2,261	3,958
Age at first intercourse (years)	16.5	16.9	17.6	18.2	17.1	17.9	18.8	20.8
Multipartner — 1 year	27.6	14.1	11.5	8.3	12.1	6.8	5.9	2.9
Intercourse (no.) — 4 weeks (m)	7.6	9.6	9.8	6.8	8.3	8.9	8.5	5.8
Homosexuality — life (%)	2.4	4.2	4.3	4.5	1.2	3.8	2.8	2.4
Intercourse with prostitutes — 5 years	4.7	3.8	3.4	1.8	—	—	—	—
IV drugs — life (%)	0.4	1.2	0.6	0.0	0.2	0.5	0.2	0.0
Condoms — life (%)	73.1	58.1	57.8	48.1	54.7	48.9	49.6	33.9
Condoms — 1 year	58.8	32.3	30.5	17.2	40.8	26.7	23.0	11.1
Monopartner	50.5	26.0	26.5	14.6	37.8	24.1	22.3	10.2
Multipartner (heterosex.)	79.0	69.0	59.7	42.7	62.4	62.0	33.3	33.1

reported in the British survey⁷. It is also similar to those found in other surveys conducted on closely related subjects in France¹⁵. Of the 20,687 questionnaires started, 632 (3.1%) were not completed. The refusal and drop-out rates did not differ significantly between interviewers. A total of 20,055 questionnaires were available for analysis, corresponding to 9,928 men (including 2,642 long questionnaires of which 1,146 were 'controls') and 10,127 women (including 2,178 long questionnaires of which 1,403 were 'controls').

For questions on sexual practices, the non-response rate varied between 0 and 4.2%, the higher figure being for questions relating to anal sex. For all the other questions, non-response rates were always below 2%, except for questions involving sexual relationships with people of the same sex, where the non-response rate was 1.1% for men but 5.9% for women ($P<0.001$). The main results are summarized in Table 1.

Initial results

The median age at the time of first sexual intercourse was 20 years for men and 22 years for women born in 1922-26, and 17 and 18 years respectively for those born in 1972-74. No increase of age at first sexual intercourse was apparent in younger people who began their sexually active life after the onset of the AIDS epidemic.

No definition of 'sexual intercourse' was provided in the questionnaire. Between 4 and 5% of people who said they lived with a partner nonetheless declared no sexual partners for the reference period. The actual distribution of the number of sexual partners over the preceding year is indicated in Table 2. The difference between men and women is much more pronounced over a whole lifetime than it is for shorter periods of time, as the total number of sexual partners during a lifetime has a mean value of 11.0 for men and 3.3 for women ($P<0.001$), these figures being respec-

tively 1.2 and 0.9 for the past year.

A small difference emerges for the number of occasions of intercourse over the past 4 weeks (8.0 for men and 7.0 for women). The proportion of people who said that they had had intercourse with at least two people during the past year differed between men and women (13.3 and 5.6% respectively, $P<0.001$). This difference between the sexes is reported in all surveys of this type^{4,16}, and is presumed to stem from both female understatement and male overstatement as well as from a greater diversity of male responses. However, the difference between the sexes seems narrower than it was 20 years ago when the ratio between men and women for the total number of partners was reported to be around 1 to 6 (ref. 8).

The proportion of multipartner subjects (at least two sexual partners over the previous year) decreases sharply with age, especially after 25 years ($P<0.001$). Of the youngest multipartner subjects (18-24 years), the proportion of those declaring at least two sexual partners at the same time (6.9% for men and 5.0% for women) is still lower than in the 35-49-year age group, where it reaches its maximum (33.7% for men and 51.7% for women). Young people change partners more frequently than older people but are less likely to have more than one sexual partner at any one time.

The proportion of multipartner subjects in rural communities (10.4% for men and 4.1% for women) was half that recorded in more built-up areas and the Paris region (18.2% for men and 10.4% for women). The rate of acquisition of new sexual partners is an important factor in the spread of AIDS. Among multipartner heterosexual people, 18.1% of men and 9.5% of women had three or more new partners during this time period, these proportions being respectively 32.2 and 17.7% for homosexual/bisexual men and women.

The proportion of subjects reporting at least one occurrence of intercourse

with a person of the same sex during their lifetime was 4.1% for men and 2.6% for women. These proportions were respectively 1.4 and 0.4% for the past 5 years and 1.1 and 0.3% over the past year. These results concur with those found in similar studies^{4,17}, but the estimates made are strongly affected by the wording of the questions. These practices are reported to be more frequent in large urban areas (4.7 times higher in Paris than in rural communities). Of the people who had had homosexual intercourse at least once, most had had intercourse with people of both sexes (82% of men and 78% of women).

Intercourse with prostitutes was found only in significant proportions among men: 3.3% over the past 5 years. This practice was much more frequent in Paris (6.2%) than in rural communities (1.7%) ($P<0.001$). The proportion of men having their first intercourse with a prostitute has dramatically decreased during the past 20 years: 10% for the men now aged 45-69, compared to only 2% for those aged 20-24.

In this type of survey it is very difficult to estimate the proportion of people taking drugs by intravenous injection, this practice being very uncommon. However, one might expect that people who regularly use drugs are the most difficult to contact, and/or most often refuse to participate in any kind of survey or to acknowledge an illegal practice. Even though 14.5% of men and 9.4% of women stated that they had taken a soft or hard drug at least once, the proportion saying they had taken an intravenous drug was very low (0.5% of men and 0.2% of women).

The proportion of subjects saying they had used a condom during intercourse at least once in their lifetime was 56.5% for men and 43.7% for women ($P<0.001$). These prevalences decreased very sharply with age, indicating the most widespread use among the youngest subjects ($P<0.001$). The usage rates over the past year were very high for the youngest subjects (aged 18-19 years): 79.8 and 48.0% for sexually active men and women, respectively. These proportions then fall gradually, indicating highly contrasting behaviour between different age groups and generations. The proportion of new condom users over the past year was significantly higher in women than in men, especially for the younger age groups (4.8% in men and 9.8% in women for 18-24 years, $P<0.05$). The condom was used for contraceptive purposes by 23.6% of men and 8.8% of women in this age group. The data also indicate that condom use is more prevalent in subjects most exposed to the risk of contamination by sexually transmitted diseases (STDs) and AIDS, that is, those

TABLE 2 Distribution of the number of sexual partners during the previous year

	0	1	2-3	4-5	6-14	15 +	Unknown	Total
Age (years)								
Men								
18-19	32.8	42.8	17.2	4.7	2.2	0.3	—	100
20-24	17.9	56.9	17.8	4.3	2.1	0.7	0.3	100
25-29	8.0	74.7	13.5	2.2	1.3	0.3	0.0	100
30-34	5.5	84.1	8.2	1.2	0.7	0.2	0.1	100
35-39	5.0	84.1	9.0	1.1	0.5	0.1	0.2	100
40-44	5.5	85.1	7.4	1.3	0.5	0.2	—	100
45-49	6.5	85.4	6.8	0.8	0.4	0.1	—	100
50-54	6.6	87.8	4.3	1.0	0.3	—	—	100
55-59	8.4	88.2	2.6	0.6	0.2	—	—	100
60-64	17.6	78.3	3.3	0.4	0.3	0.1	—	100
65-69	24.2	73.8	1.8	0.1	0.1	—	—	100
Total	11.1	77.5	8.7	1.6	0.8	0.2	0.1	100
Women								
18-19	41.2	49.6	7.7	1.1	0.4	—	—	100
20-24	20.3	69.7	8.7	1.0	0.3	—	—	100
25-29	9.0	83.6	6.3	0.5	0.5	0.1	—	100
30-34	4.9	89.5	5.0	0.4	0.2	—	—	100
35-39	6.0	89.0	4.2	0.4	0.4	—	—	100
40-44	8.2	88.2	3.4	0.2	—	—	—	100
45-49	9.9	85.8	3.6	0.5	—	—	0.2	100
50-54	15.3	82.1	2.6	—	—	—	—	100
55-59	24.9	73.9	1.2	—	—	—	—	100
60-64	32.5	66.8	0.6	—	—	—	0.1	100
65-69	44.3	55.6	—	—	—	—	0.1	100
Total	17.3	78.0	4.1	0.4	0.2	—	0.0	100

All values in per cent.

with several homosexual or heterosexual partners. Around 75% of multipartner homosexual men aged 18-44 years, and more than 65% of men and 50% of women with multiple heterosexual partners, were found to have used a condom at least once over the past year, but this still leaves a high proportion who do not: one-third of the heterosexual men, half of the heterosexual women and one-quarter of the homosexual/bisexual men did not use condoms at any time during this period.

One feature of our study that is different from any other is the collection of detailed data on condom use during the last occasion of intercourse. Overall, 18% of men and almost 11% of women used a condom during last intercourse. A multivariate analysis indicates that the most important determinants of condom use among those with multipartners are age of the subject (with a much higher use among young people), type of partner (2.5 times higher usage rate with casual partners than with regular ones), length of the relationship (for women, 3.1 times higher condom use for the first intercourse with a new partner) and perceiving that one was personally at risk of contracting AIDS. However, the situation regarding this last variable is complex because among men the reverse is true: condom use is higher in those who are not concerned about getting AIDS. This observation supports the

hypothesis that people at high risk have changed their behaviour and are using condoms more often, and therefore no longer feel themselves to be personally at risk^{5,18}.

Subsequent analyses

The results presented here provide a vast amount of information on the sexual behaviour of the population in France. The most striking finding is the relatively high level of condom use in young people and in those most exposed to the risk of contamination by STDs or by AIDS, although a considerable proportion of these people still do not use condoms, especially with new partners. A more specific survey is now being conducted among young people aged 15-18 years.

The available data should reveal which people protect themselves against the risk of contamination and which do not, taking account of the different types of sexual partners and the practices performed with each of them. Moreover, our study was set up not only to describe the characteristics of these people, but also to try to understand what psychological and sociological mechanisms are at play. These aspects were explored in depth in our questionnaire¹¹ and we are now analysing them. They include, for example, social norms relating to sexual behaviour; influence of the family environment; strategies for finding sexual partners; verbal communication with a

new sexual partner; attitudes about body fluids and so on.

Apart from a few exceptions, sexual behaviour until very recently remained one of the few areas in which there was no systematic information available from surveys conducted on representative samples of the general population in developed countries¹². The knowledge provided by cross-sectional surveys similar to the one we report here is essential both to optimize strategies for preventing STDs and AIDS and to predict the spread of the AIDS epidemic more accurately.

The rate of transmission of STDs and AIDS in a given population depends on a large number of behavioural variables: the most important of these are the rate of acquisition of new partners, the type of sexual practice and patterns of sexual 'mixing' such as age-dependent partner choice and mixing between populations with differential disease prevalence¹⁹. The level of protection currently used is insufficient to halt the spread of the HIV epidemic. Small-scale studies, repeated at two-yearly intervals, are planned to monitor future changes in sexual behaviour. Quantitative surveys of this kind have their limitations, however. Qualitative studies are also called for to reach marginal groups of the population and to explore certain kinds of behaviour in depth. Only by combining the two approaches will it be possible to obtain sufficient information to define effective strategies to prevent the spread of STDs and AIDS.

Since submission of this manuscript, the survey which follows on page 410 has been completed²⁰. □

1. AIDS 6, 243-247 (1992).
2. Frank, O. *Recherches sur les comportements sexuels et la santé génésique: perspectives actuelles et priorités futures* (Human Reproduction Program, World Health Organization, 1990).
3. Sundet, J. M., Magnus, P., Kvale, I. L., Gronnesby, J. K. & Bakkeiteig, L. S. *Health Policy* 13, 160-167 (1989).
4. Melbye, M. & Biggar, R. J. *Am. J. Epidemiol.* 135, 593-602 (1992).
5. McQueen, D. V. & Uitenbroek, D. G. *Health Educ. Res.* 7, 47-53 (1992).
6. Aldhous, P. *Science* 257, 25 (1992).
7. Wellings, K. et al. *Nature* 348, 276-278 (1990).
8. Simon, P., Gondonneau, J., Mironer, L. & Dourien-Rollier, A. M. *Rapport sur le comportement sexuel des français* (Julliard et Charron, Paris, 1972).
9. ACSF Investigators *Bull. Method. Sociol.* 35, 46-54 (1992).
10. ACSF Investigators *AIDS* 6, 315-323 (1992).
11. Bajos, N., Spira, A. & ACSF *Sciences Sociales et Santé* 1X, 58-68 (1991).
12. Catania, J., Gibson, D., Chitwood, D. & Coates, T. *Psychol. Bull.* 108, 339-362 (1990).
13. Upchurch, D. M. et al. *Am. J. Epidemiol.* 134, 1159-1166 (1991).
14. Czaja, R. *Int. Quart. Commun. Health Educ.* 8, 23-32 (1988).
15. Leridon, H. & Toulemon, L. *Population* 2, 777-812 (1991).
16. Lagrange, H. *Population* 2, 249-278 (1991).
17. Rogers, S. M. & Turner, C. F. J. *Sex Res.* 28, 491-519 (1991).
18. Pollak, M. in *Assessing AIDS Prevention* (eds Paccaud, F., Vader, J. & Gutzwiller, F.) 305 (IUMSP, Lausanne, 1992).
19. Anderson, R. M. & May, R. M. *Nature* 333, 514-519 (1988).
20. Johnson, A. M. et al. *Nature* 360, 410-412 (1992).

Sexual lifestyles and HIV risk

Anne M. Johnson, Jane Wadsworth, Kaye Wellings, Sally Bradshaw and Julia Field

Britain's first large, national survey of sexual attitudes and lifestyles will allow improved estimates of the magnitude of the HIV epidemic in Britain and should lead to better strategies for prevention.

BETWEEN May 1990 and December 1991, we undertook a random-sample survey of sexual attitudes and lifestyles among 18,876 men and women aged 16–59 living in England, Wales and Scotland. The methodology was established through an extended period of feasibility work which has been described previously¹. Our survey is designed to provide baseline estimates for use by those concerned with sexual behaviour, health and reproduction. In particular, we wish to contribute to understanding the epidemiology of human immunodeficiency virus (HIV) and sexually transmitted diseases (STDs), to assess temporal changes in behaviour and to assist in the design of prevention strategies. As with all self-reported data, there are problems of reliability², but studies in this particularly sensitive area have also met political opposition³. Large sample surveys are gradually being undertaken in other countries^{2,4,5} but the French survey on page 407 of this issue⁴ and our British survey are two of the first to be completed.

Methods to estimate the current and future spread of HIV in Britain include mathematical models⁶, which require information on the frequency and pattern of sexual partner change in the population; direct methods, which combine measures of HIV prevalence in selected populations with estimates of the size of such populations; and indirect methods, which derive the total infected from the number of people diagnosed as HIV-antibody positive and the proportion in the population known to have been tested for HIV antibody^{7–9}. Previous estimates have relied on imprecise data as there were no measures of behaviour patterns in large, representative population samples^{7,8}. In the past year, advances in understanding the extent of HIV infection in Britain have been made through a programme of unlinked anonymous HIV prevalence monitoring among women attending antenatal clinics, people attending STD clinics, newborn infants and injecting drug users¹⁰.

We sampled electoral wards stratified to be representative of urban, rural and metropolitan districts. Within each area, addresses from the post office's postcode address file were selected systematically from a random starting point. Each household was approached by a trained interviewer who invited a randomly

selected individual between the ages of 16 and 59 to participate. Among potentially eligible addresses, no contact was achieved after at least four calls at 3.5% and complete refusal of information was met at 6%. After adjusting for the estimated proportion of eligible households at these addresses, the response rate was 65%. Among households in which an eligible resident could be identified and selected, a completed interview was achieved in 72%. We weighted the sample to adjust for household size and differential response rate between regions to obtain population estimates of behaviours. All estimates reported here are based on weighted data. The achieved sample was broadly representative of the age, marital status and social-class structure of the population of England, Wales and Scotland^{11,12}. Non-responders were more likely to be men and older people.

Each interview was carried out in two modes. First, face-to-face questions covered health, sex education, family background, attitudes to sex, age at first heterosexual intercourse, homosexual and heterosexual attraction and experi-

ence. More sensitive questions were written on show cards requiring only a number or letter as a response. Second, a self-completion questionnaire included questions on sexual practices, numbers of heterosexual and homosexual partners, history of injecting drug use and attendance at an STD clinic.

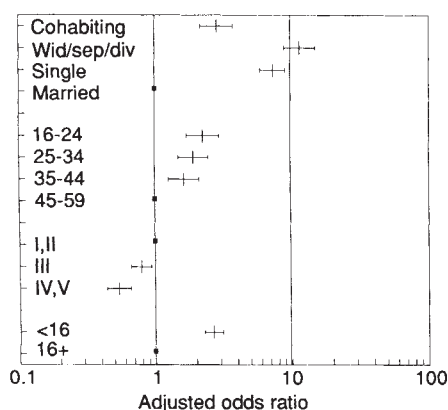
Clearly evident is the greater tendency of younger respondents, particularly men, to report multiple partners over recent time intervals (Table 1). The proportion of respondents reporting two or more heterosexual partners in the past year increased from 1.8% of women over 45 to 26.9% of men aged 16–24 (even though this is also the group with the greatest proportion having no partners in the past year).

In addition to age, a number of interdependent demographic and behavioural variables might be expected to influence numbers of partners. In a series of logistic regression models, we examined the simultaneous effects of age, marital status, social class and age at first intercourse on the likelihood of two or more partners in the past year (see figure), three or more partners in the past 5

TABLE 1 No. of heterosexual partners by age and gender

Age group	16–24	25–34	35–44	45–59	All	16–24	25–34	35–44	45–59	All
Lifetime										
None	20.4%	3.1%	1.9%	1.5%	6.6%	20.7%	2.1%	0.7%	1.5%	5.7%
One	16.3%	15.0%	20.5%	30.5%	20.6%	27.0%	30.8%	40.7%	57.7%	39.3%
Two	9.8%	9.2%	10.7%	12.6%	10.6%	14.7%	18.3%	17.7%	16.6%	16.9%
3–4	19.4%	18.2%	17.1%	18.9%	18.4%	18.8%	22.5%	18.5%	12.9%	18.2%
5–9	17.9%	23.1%	20.9%	15.8%	19.4%	14.1%	16.7%	13.9%	7.4%	13.0%
10+	16.2%	31.4%	28.9%	20.8%	24.4%	4.6%	9.7%	8.5%	3.8%	6.8%
99th centile	45	86	75	100	75	18	25	28	20	25
Mean	5.3	10.3	10.2	13.6	9.9	2.8	4.3	3.7	2.6	3.4
Variance	98.1	720.5	2838.5	22093	6575.1	25.9	315.0	42.2	196.7	165.3
Past 5 years										
None	20.6%	4.6%	4.5%	5.7%	8.7%	20.8%	3.1%	3.3%	11.3%	9.1%
One	20.1%	51.7%	72.6%	80.6%	56.5%	33.1%	67.6%	82.4%	81.9%	67.4%
Two	11.8%	12.4%	9.4%	6.4%	10.0%	16.3%	14.3%	9.0%	4.9%	11.0%
3–4	20.2%	15.9%	7.5%	4.7%	12.0%	16.9%	10.3%	4.3%	1.7%	8.1%
5–9	16.1%	9.6%	4.2%	1.9%	7.9%	10.5%	3.8%	0.9%	0.2%	3.6%
10+	11.2%	5.8%	1.7%	0.70%	4.8%	2.5%	0.8%	0.2%	0.04%	0.8%
99th centile	35	25	12	6	22	15	8	5	3	8
Mean	4.2	3.1	1.7	1.3	2.6	2.3	1.7	1.2	1.0	1.5
Variance	61.6	74.6	5.1	3.9	37.2	18.4	3.3	0.8	0.3	4.8
Past year										
None	26.9%	8.6%	6.8%	10.8%	13.1%	23.9%	6.7%	7.4%	19.3%	13.9%
One	46.2%	76.9%	84.1%	83.8%	73.0%	60.5%	86.8%	88.4%	78.9%	79.4%
Two	14.3%	8.6%	6.1%	4.2%	8.2%	10.0%	4.9%	3.3%	1.6%	4.8%
3–4	9.1%	4.1%	2.1%	1.1%	4.1%	4.5%	1.3%	0.8%	0.2%	1.6%
5+	3.5%	1.9%	0.8%	0.1%	1.5%	1.0%	0.3%	0.1%	0	0.4%
99th centile	10	5	4	3	6	7	3	2	2	3
Mean	1.4	1.2	1.1	1.0	1.2	1.0	1.0	1.0	0.8	1.0
Variance	5.2	8.9	1.1	0.3	4.0	1.8	0.3	0.2	0.2	0.5
<i>N</i>	1,984	2,167	2,051	2,182	8,384	2,246	2,899	2,576	2,771	10,492
Missing	45	65	77	150	337	49	101	94	188	432

Partners defined as members of the opposite sex with whom the respondent had had vaginal or anal intercourse or orogenital contact.



Adjusted odds ratios for two or more partners in the past year among men. Results of the logistic regression models are presented as odds ratios and their 95% confidence intervals after adjustment for all other factors in the model. An odds ratio of 1 implies equivalence of risks, hence where the 95% confidence interval for any factor includes 1, the results are consistent with there being no excess risk. 13.8% of men reported two or more partners in the past year. Single men were 7.3 times more likely to report multiple partners than those who were married. Men who were cohabiting showed an odds ratio of 2.8 for multiple partners compared with those who were married. I-V, social class; bottom two lines, age of first sexual intercourse.

years and ten or more partners ever. We found that influences on multiple partnerships were similar for men and women. After controlling for other variables in the model, the probability of reporting two or more partners in the past year was significantly increased among the unmarried (including cohabitantes); those who reported first heterosexual intercourse before age 16; and those in the two highest social classes.

In a similar model for reporting ten or more partners over a lifetime (so far), a slightly different picture emerged. 'Lifetime' partners reflect both recent and past behaviour, and are influenced by the number of sexually active years. The proportion reporting ten or more partners is higher among those aged 25-44 than among those over 45, though lower among men aged 16-24 who have become sexually active more recently. This age effect was sustained in our model.

Although the youngest respondents were the most likely to report more partners in recent time intervals, partner accrual does not cease in the teens and twenties. The differences between recent and lifetime behaviour reflect the influences of both birth cohort and life stages. Those of higher socio-economic status were more likely to report larger numbers of partners, an effect more marked among men than among women and maintained when age at marriage was included. Early sexual experience may also influence long-term sexual behaviour, as those who experienced first coitus before age 16 were the most likely to report larger numbers of partners.

We note the extreme variability in the

number of reported heterosexual partners: 1% of men reported 16% of all partners in the past 5 years (women 12%), a finding important to STD transmission. The mean and variance are strongly influenced by the few people reporting a very large number of partners: for men aged 45-59, for example, the mean lifetime number of reported partners is 13.6 with a variance of 22093. Such extreme skewness may be unduly influenced by memory error, particularly among people with long and varied sexual careers, making the mean an unstable, potentially unreliable summary statistic¹. As in other surveys, men report more partners than women. We have previously discussed possible reasons for this¹³.

Men and women were asked about homosexual behaviour. The results presented are restricted to men. Questions ranged from same gender sexual experience as defined by the respondent, through experience involving genital contact of different kinds, to reporting of homosexual partners (defined as a male partner with whom anal sex or oro-genital sex or other forms of genital contact had occurred). Two of the measures used here, based on self-completion questions, are the proportion of men reporting any experience (an all-inclusive estimate) and those men reporting homosexual partners.

Overall, 6.1% of men reported any homosexual experience; 3.6% reported ever having a homosexual partner and 1.4% reported a partner in the past 5 years (Table 2). Thus, the 'prevalence' of homosexual experience depends on the definition of what constitutes such an experience and over what time period it is measured. Our results are consistent with those from other recent studies in Europe and the United States^{14,15} despite the absence of studies of equivalent design and size, and despite presumed cultural differences. Recent experience of a homosexual partnership (past 5 years) was most frequent among men under 35 (1.8%), whereas lifetime experience of a homosexual partnership peaked in those aged 35-44 (4.8%).

TABLE 2 Sexual contact between men

	Greater London	Great Britain
Any homosexual experience	11.9 (9.7, 13.7)	6.1 (5.6, 6.7)
1+ homosexual partner ever	8.6 (6.9, 10.5)	3.6 (3.2, 4.0)
1+ homosexual partner in past 5 years	4.6 (3.4, 6.0)	1.4 (1.2, 1.7)
1+ homosexual partner past year	3.5 (2.5, 4.7)	1.1 (0.8, 1.3)
Base	1,125	8,337

All values in % (95% confidence interval).

There were very striking regional differences in reporting of homosexual partnerships: 11.9% of men living in greater London reported any homosexual experience and 8.6% reported ever having a homosexual partner, and close to 1 in 20 men in greater London reported a homosexual partner in the past 5 years, consistent with the likelihood of homosexual men migrating to London.

Only 0.8% of men and 0.4% of women reported ever injecting non-prescribed drugs, these figures falling to 0.4% of men and 0.3% of women injecting in the past 5 years. Highest rates for ever injecting were among those living in greater London (2.1% of men, 0.8% of women). There are age differences, reports of injecting being almost entirely limited to those under 45. For the population aged under 45, the proportion reporting ever injecting was 1.0% for men and 0.5% for women. Of those with a history of injecting non-prescribed drugs, 53% reported ever sharing needles and syringes, while no sharing was reported by those using prescribed drugs. Although these estimates are minima, applying these rates to the population of England and Wales gives an estimate of 100,000 injecting in the past 5 years and 175,000 ever. These figures are of a similar order of magnitude to a previous estimate⁹ of 120,000 injecting drug users in England and Wales based on scarce data from surveys of drug users and government reports.

TABLE 3 Health service use and sexual behaviour in past five years

No. of homosexual partners		0	1	2	3-4	5+
Attended STD clinic	Men	3.1% (7,505)	7.9% (49)	9.4% (11)	32.0% (18)	51.4% (41)
	Women	0.3% (510)	1.2% (6,503)	4.1% (1,062)	8.0% (771)	15.7% (431)
No. of heterosexual partners		0	1	2	3-4	5+
Attended STD clinic	Men	7.5% (357)	0.7% (4,490)	2.9% (789)	4.5% (958)	13.7% (1,017)
	Women	0.3% (510)	1.2% (6,503)	4.1% (1,062)	8.0% (771)	15.7% (431)
No. of heterosexual partners		0	1	2	3-4	5+
Has child under 5	Men	0.06% (701)	20.6% (4,518)	16.7% (801)	15.9% (966)	12.7% (1,023)
	Women*	0.8% (626)	33.8% (4,660)	27.2% (978)	19.5% (762)	17.0% (437)

* Women aged 16-44. Numbers in parentheses, bases for percentages.

Current data on the prevalence of HIV infection are based on samples obtained from people attending National Health Service clinics (STD, antenatal), drug users attending addiction clinics or needle-exchange programmes and from volunteer samples (homosexual men, drug users, and so on). An understanding of how the behaviour patterns of people using these services compares with those of the whole population is necessary before data from the former can be extrapolated.

Our data show that a high proportion of people with multiple partners reported STD clinic attendance. More than one in seven of those with five or

use was associated with a significantly increased likelihood of clinic attendance.

In univariate analysis, the probability of childbirth in the past 5 years decreased with increasing numbers of partners. In a logistic model controlling for age and marital status, women who reported childbirth in the past 5 years were significantly less likely to have more than one partner than those who did not (data not shown). When an identical model was constructed for men, the effects were broadly similar, except that men who had become fathers in the past 5 years were no more or less likely to have had only one partner in that time. Thus women attending antenatal clinics were more likely to be monogamous than other women of a similar age, but their male partners may not differ from other men in this respect. Such factors need to be considered in interpreting results of anonymous testing programmes.

Respondents reported whether they had had an HIV test in the past 5 years and whether this was in connection with blood donation, pregnancy, insurance/travel or 'other' (unspecified) reasons (Table 4). The last category thus included those who had sought testing. Overall, more than 13% of the sample reported that they had had an HIV test. The commonest reason for testing was blood donation (7.5% of men, 6.2% of women) but over 4% of men and nearly 3% of women had had a test for reasons other than blood donation, pregnancy and insurance/travel. Rates of testing declined in those aged 45 or more.

More than one in five men (and one in four women) with five or more heterosexual partners in the past 5 years reported having an HIV test, and one in ten had done so for reasons other than for blood donation and so on. Among men with homosexual partners in the past 5 years, the proportion rose to more than four out of ten with more than one in four seeking testing for an 'other' reason. For those injecting non-prescribed drugs, nearly half had been tested. All these rates are in significant excess of those of the overall population, suggesting that a considerable proportion of those with high-risk behaviours have chosen to undertake HIV testing.

In this large random-sample survey, we have been able to demonstrate some of the variability in sexual lifestyles in Britain, and to improve understanding of the demographic correlates of behaviour patterns. The high rate of STD clinic attendance by those with multiple partners, male homosexual contact and history of injecting drug use emphasizes the importance of these clinics as a focus for promoting sexual health as advocated in a recent government White Paper (policy document)¹⁶. Our study indicates that many of those at high risk of HIV

infection have perceived this risk and have already undergone HIV antibody testing, but that more than half remain untested. This measure gives an indication of the level of undiagnosed HIV infection in the population.

Although we are aware of the many caveats which must be applied to the interpretation of self-reported data, and that estimates of socially censured behaviours have to be regarded as minima, many of our results form a consistent picture with findings from independent data sources or from volunteer samples of those at high risk. So far, we have addressed only those findings essential to public-health strategy and to estimates at the magnitude of the HIV epidemic; a more detailed exposition of methodologies and results is in preparation. Our study provides a backdrop against which research in more focused populations at high risk using volunteer samples can be assessed. The research instrument itself, which has involved considerable development, can be applied to other population subgroups to obtain comparable data. Repeat surveys of specific data subsets, in conjunction with other aspects of health, may allow assessment of future changes in behaviour patterns. □

Anne M. Johnson and Sally Bradshaw are in the Academic Department of Genito Urinary Medicine, University College London Medical School, London W1N 8AA; Jane Wadsworth and Kaye Wellings are in the Academic Department of Public Health, St Mary's Hospital Medical School, London W2 1NY; Julia Field is at Social and Community Planning Research, 35 Northampton Square, London EC1V 0AX, UK.

TABLE 4 Behaviour, childbirth and HIV antibody testing

		Any reason	'Other' reason*
5+ heterosexual partners in past 5 years	Men	21.0% (1,017)	10.2% (1,016)
	Women	26.6% (441)	8.9% (441)
1+ homosexual partners in past 5 years	Men	43.0% (118)	27.0% (118)
Ever injected non-prescribed drugs	Men	41.5% (58)	31.4% (58)
	Women	61.9% (35)	48.6% (35)
Has child under 5	Men	11.7% (1,334)	3.2% (1,333)
	Women	21.7% (2,042)	2.1% (2,036)
All respondents	Men	13.2% (7,577)	4.2% (7,552)
	Women	13.7% (9,463)	2.9% (9,426)

* Excludes tests for blood donation, pregnancy or insurance/travel. Numbers in parentheses, bases for percentages.

more heterosexual partners in the past 5 years had attended a clinic in that time (Table 3) and more than one in five of those reporting ten or more lifetime partners had ever attended. Among men reporting homosexual partners, more than half with five or more partners in the past 5 years had attended a clinic in the same time period.

We have also constructed a series of logistic regression models to assess the simultaneous effects of age, marital status, numbers of heterosexual partners, homosexual contact and non-prescribed drug injection, on the likelihood of attendance at an STD clinic in the past 5 years (data not shown). After adjustments for other factors in the model, age and marital status influenced the likelihood of clinic attendance. Increasing numbers of heterosexual partners exerted a strong effect for men and women but homosexual partnerships for men only, confirming the strong association between behaviour and clinic attendance. For women, injecting drug

- Wellings, K. *et al.* *Nature* **348**, 276–278 (1990).
- Wadsworth, J. & Johnson, A. M. *J. R. Statist. Soc. A* **154**, 367–370 (1991).
- Maaddox, J. *Nature* **341**, 181 (1989).
- ACSF investigators *Nature* **360**, 407–409 (1992).
- Carballo, M., *et al.* *J. Sex Res.* **26**, 287–299 (1989).
- Anderson, R. M. & May, R. M. *Nature* **333**, 514–519 (1988).
- Short term prediction of HIV infection and AIDS in England and Wales 48–52 (Department of Health, Welsh Office, 1988).
- CDR 1–12 (1990).
- Hillier, H. in *Short-term prediction of HIV infection and AIDS in England and Wales* 48–52 (Department of Health, Welsh Office, 1988).
- PHLS AIDS Centre, *et al.* *CDR* **1**, R69–R76 (1991).
- General Household Survey (HMSO, London, 1989).
- OPCS Population Projections 1985–2025. Series PP2 No.15 (OPCS, London, 1992).
- Johnson, A. M., *et al.* *Nature* **343**, 109 (1990).
- Rogers, S. M. & Turner, C. F. *J. Sex Res.* **28**, 491–519 (1991).
- Sundet, J. M. *et al.* in *The global impact of AIDS* (eds Fleming, A. F., *et al.* 53–56 (Liss, London, 1988).
- The Health of the Nation. A Strategy for Health in England* (HMSO, London, 1992).

ACKNOWLEDGEMENTS. We thank the participants, interviewers and the field staff at Social and Community Planning Research for fieldwork and data processing. We thank Michael Adler, Roy Anderson, Roger Jewell and David Miller for advice; and Kay Stratton and Carol Morgan for manuscript preparation. Supported by the Wellcome Trust. A longer version of this manuscript, containing the details of the logistic model, is obtainable on request from A. M. J. or from the *Nature* London editorial office.

A distant candle

The discovery of a supernova at a redshift of one-half holds the promise of a new determination of the cosmic density parameter. But promise and practice are some way apart.

DECIDING whether the Universe will expand forever or instead one day fall back on itself is very easy. All one needs to do is find two objects of identical luminosity, one nearby and one farther away, and measure their apparent brightnesses. For uniform cosmological models in which the single unknown parameter is Ω , the ratio of density to the critical density required for closure, there is a family of curves relating brightness to redshift, so the accurate measurement of two points on that curve suffices to pick out one value of Ω . If it is less than one, the Universe expands forever; if it is greater than one, the Universe must eventually collapse.

Astrophysical objects of fixed brightness are not easy to find, but there is one good possibility. A type Ia supernova occurs when a white dwarf blows up, a catastrophe triggered by the gradual accretion of material from a companion star onto the surface of the white dwarf until the internal density rises to the point where gravitational pressure overwhelms the ability of the degenerate core to withstand it. A thermonuclear event ensues, blowing the white dwarf to pieces, and the nature of this explosion ought to depend on the physics of white dwarfs and little else. A type Ia supernova ought therefore to be the same no matter where it occurs, so that it can reliably be called a "standard candle", of the sort that cosmologists have long hoped to find.

This is all well known, but its significance has come to the fore lately because of the discovery, by Saul Perlmutter and colleagues at the Lawrence Berkeley Laboratory, of a type Ia supernova in an anonymous galaxy whose redshift was subsequently established, by Karl Glazebrook of the University of Durham, to be $z = 0.457$. This is the most distant supernova yet seen, and the hope is that it will act as a calibration point on the cosmological brightness versus redshift curve at a large enough redshift that curves for different values of Ω (say, 0.2 and 1.0) will be easily distinguished.

Simple enough but, as always with cosmological measurements, things are not as simple as they seem. The fact that this object is called a type Ia supernova implies, of course, that there are other types of supernova, so the first thing for Perlmutter and colleagues to establish is that the object they have seen is what they think it is. Type I supernovae are distinguished from Type II by the absence of hydrogen lines in the spectrum, which is easy enough to check, but type I supernovae are then split into different sorts by more subtle spectral analy-

sis. Type Ib supernovae lack a certain Si absorption line and, at late stages of development, are dominated by oxygen rather than iron features, but in early outburst can be hard to distinguish from Type Ia supernovae. They are, however, substantially dimmer, so an astronomer mistaking a type Ib for a type Ia would think the Universe much bigger than it is.

Supposing the supernova in question to have been correctly labelled as a type Ia, there are still plenty of shoals to navigate. By the brightness of a supernova, which is the number the cosmological brightness-redshift curve has to pass through, is generally meant the peak brightness, which occurs a few days after outburst. Perlmutter *et al.* did not catch their distant supernova at its peak, but have been tracking its falling brightness and attempting to extrapolate thereby when the peak occurred and how bright it was. But any such extrapolation is reliable only to the extent that this type Ia is like every other type Ia, and it is precisely this kind of assumption one would like to avoid, as far as possible, in this exercise.

Moreover, local type Ia supernova brightness curves are most similar, and best studied, in the optical B-band (a blue part of the spectrum), whereas the supernova at $z = 0.457$ has been measured in the infrared R-band. Light curves are not identical in different wavelength bands, and there is an offset in the moment of peak brightness in different bands. But here is a piece of luck: the R-band, for an object at a redshift of about one-half, is equivalent to the B-band at the source, so that Perlmutter *et al.* are in fact measuring precisely the light-curve they need to measure to make the best comparison with the body of evidence on local type Ia supernovae.

Set against this, however, is the problem that the observed colours of any astronomical object are modified — reddened — by dust and gas in its local environment. If it were true that type Ia supernovae are precisely identical, then the effects of reddening could be subtracted out by comparing observations in different wavelength bands. But Perlmutter *et al.* have only a partial supernova lightcurve at a single wavelength, and they want moreover to avoid undue reliance on the assumption that their supernova is the same as everyone else's. In local supernova surveys it is possible, for example, to include only events that occur in elliptical galaxies, for which reddening due to dust is presumably slight, but if one is interested in supernovae at moderate redshifts, asking for a

precise identification of the type of galaxy they are in is asking for a lot.

Setting these mere observational difficulties aside, there remains the most vexing question of all: how identical are type Ia supernovae? To say that they happen when a white dwarf reaches a certain critical core density implies that they should all be the same, but the empirical fact is that local type Ia supernovae are not precisely the same, even allowing for observational uncertainties. The colours at maximum vary slightly, and there are some 'peculiar' type Ia events, which show a variety of odd photometric and spectroscopic features.

This is hardly unreasonable. White dwarfs are a homogeneous class of objects, compared, for example, to main sequence stars, but they can have slightly different masses, interior temperatures, compositions, rotation rates and so on. All of these factors may alter, in subtle ways, the nature of a white dwarf explosion. Compositional differences, because they pertain mostly to the outer layers of the white dwarf, may have a marked effect on the light-curve near-peak brightness, because one is then observing the outermost edge of the ejected material. Theoretical models of type Ia explosions suggest two broad possibilities: the white dwarf can be dispersed by a nuclear deflagration wave or by a supersonic detonation wave, the time-scale and energy release being different between the two cases.

What this all means, as Perlmutter *et al.* certainly recognise, is that many moderate-redshift type Ia supernovae will have to be detected. The optimistic view is that when 20 or 30 such events have been recorded, they will cluster around a single cosmological brightness-redshift curve in such a way that all the scatter can be put down to observational uncertainties; this will imply that all type Ia supernovae are sufficiently similar for a value of Ω to be determined. The pessimistic view is that there is some inescapable systematic variation in the characteristics of type Ia supernovae, so that as Perlmutter *et al.* search at higher redshift they will preferentially detect the intrinsically brighter events, which will not be a truly representative subsample of the whole population. And this will create a bias in their estimate of Ω , which is the problem observational cosmologists have been struggling with for decades. But the only way to find out what will happen is to undertake the necessary observations, and that is what Perlmutter *et al.* are undoubtedly planning to do.

David Lindley

An uplifting experience

David Rind

PROJECTIONS of a rapid warming of the climate over the next century, due to increased greenhouse gases, have focused attention on the last time the Earth experienced very warm climates, during the Tertiary Period (63–2.5 million years ago). The end of that era, and the onset of the modern alternation between glacial and interglacial climates, has been related to the rapid development of the great mountain belts,

glaciers or to greater storminess, an isostatic (buoyant) response will raise the elevation of individual peaks, giving the appearance of uplift while in fact lowering the mean elevation relative to sea level. There is no obvious tectonic reason for widespread rapid uplift in the late Cenozoic when, according to T. Volk (New York University), spreading rates had decreased. Uplift probably occurred over a longer time frame, of

the long-term carbon cycle attempt to balance CO₂ sources, chiefly volcanic emissions whose change with time is estimated from sea-floor spreading rates, with sinks such as silicate weathering or organic-carbon burial (R. Berner, Yale University). Many of these parameters are not well known. Additional estimates of CO₂ levels come from the ¹³C content of palaeosols or marine organic material, both of which appear to be affected by the prevailing gaseous CO₂ concentration. Numerous assumptions apply here as well, and the range of estimated CO₂ levels for the Tertiary varies from seven times pre-industrial



High peaks in Nepal, south of Everest. Cause or effect of climate change? Or neither? (Photo by David Paterson.)

particularly the Himalaya. A recent mini-conference on *Uplift, Erosion and Climate** brought together various protagonists to argue out the cases for and against a causal connection.

The simplest story^{1,2} is that the rapid development of mountains during the past 5–10 million years started the ice ages. Erosion and associated chemical weathering would have drawn carbon dioxide out of the atmosphere to start the cooling³. The reduced temperatures might then have slowed down the rate of weathering, so stabilizing the CO₂ level. As acknowledged by all of the participants at the meeting, many uncertainties surround the observations and their interpretation, but the debate could clearly be broken down into a number of distinct issues.

Did rapid uplift really occur? Apparently not. As emphasized by P. Molnar (Massachusetts Institute of Technology), much of the evidence suggesting rapid uplift can be explained more easily in terms of the colder climate of the Late Cenozoic⁴. A colder climate produces glaciers and alters botanical distributions, making it appear as if elevation was increasing; in the case of Tibet, tectonically driven northward movement over this time would also have produced cooling. Furthermore, with increased erosion, due either to

some 50 million years, in the Himalayan region. But there may have been some acceleration 8 million years ago associated with a sudden increase in the intensity of monsoons (W. Prell, Brown University).

Did increased erosion occur in the later Cenozoic? Probably. Increases over the past 50 million years in the seawater ⁸⁷Sr/⁸⁶Sr ratio recorded by marine carbonates (D. DePaolo, University of California at Berkeley) imply an increase in the volume of continental weathering, although changes in source composition and path to the ocean are also possible. Greater physical weathering might be expected in the colder climate and with gradual uplift in southern Asia. The strontium changes are assumed to imply increases in the chemical weathering of silicate rocks, although this is harder to prove: it presupposes that there has been an increase in the volume of weathered rock, and that the strontium is not coming from carbonates. The idea that weathering slows as temperatures cool, owing to reduced reaction rates, so stabilizing the CO₂ level⁵, is in general conflict with this approach, which emphasizes the importance of physical weathering in increasing the surface area available for chemical reactions.

Did CO₂ levels decrease during the Cenozoic? This is certainly the general assumption, but the evidence for it is pretty weak. Theoretical models of

levels to no difference⁶. We do not really know what CO₂ concentrations were throughout the Tertiary; recent work, in fact, suggests that values in the late Pliocene (3.2 million years ago) were no higher than they were in pre-industrial times⁷ (M. Raymo, Massachusetts Institute of Technology), despite evidence indicating that polar climates were much warmer.

Were higher CO₂ levels responsible for the warm climates in the Tertiary? Again, this is the general assumption, yet there is a considerable discrepancy between the climates reconstructed from geochemical and other data for this period and those predicted from models of an atmosphere with increased CO₂. Tertiary climates as deduced from oxygen-isotope analysis and pollen data feature extreme warming at polar latitudes (of the order of 15 °C), with little temperature change in the tropics. In contrast, models for increased CO₂ produce substantial tropical warming as well, with increased water vapour levels

1. Ruddiman, W. F. & Kutzbach, J. E. *J. geophys. Res.* **94**, 18409–18427 (1989).
2. Raymo, M. E. & Ruddiman, W. F. *Nature* **359**, 117–122 (1992).
3. Raymo, M. E. *et al. Geology* **16**, 649–653 (1988).
4. Molnar, P. & England, P. *Nature* **346**, 29–34 (1990).
5. Berner, R. A. *et al. Am. J. Sci.* **283**, 641–683 (1983).
6. Berner, R. A. *Nature* **358**, 114 (1992).
7. Raymo, M. E. & Rau, G. *Eos* **73**, 95 (1992).
8. Rind, D. & Chandler, M. *J. geophys. Res.* **96**, 7437–7460 (1991).
9. Raymo, M. E. *et al. Paleoceanography* (in the press).

* *Uplift, Erosion and Climate*, Lamont Doherty Geological Observatory, 9–10 November 1992.

providing a strong positive feedback. As reported at the meeting, our model experiments⁸ can reproduce the extreme high-latitude temperature amplification of the reconstructions only by increasing ocean heat transport, a process by which sea ice becomes the prime positive feedback. The increased ocean heat transport might have come about through altered surface wind stress attributable to a change in topography, or through increases in deep-water production, perhaps associated with ocean-sill changes. Raymo noted that there is now some evidence that North Atlantic Deep Water production was greater during the late Pliocene⁹.

Where does this leave us? The participants generally agreed that although gradual uplift undoubtedly occurred in some regions, perhaps leading to increased erosion, the effects of these processes on climate are still debatable. We do not know what the CO₂ levels were in the Tertiary, nor whether higher CO₂ was in fact responsible for the warmth of those times. The question of the applicability of Tertiary climates as analogues for future trace gas-induced warming remains an open one. □

David Rind is at the Goddard Space Flight Center, Institute for Space Studies, New York, New York 10023, USA.

EVOLUTIONARY BIOLOGY

What the sperm count costs

Linda Partridge and Paul H. Harvey

DISSECTING the sex life of the nematode worm *Caenorhabditis elegans* has already provided surprises for biologists interested in life-history theory. In a report on page 456 of this issue¹, Van Voorhies throws another spanner in the works by demonstrating that the costs of producing sperm are not as negligible as we might have thought.

C. elegans individuals are normally self-fertilizing hermaphrodites, although the occasional male crops up from time to time. Last year's big surprise was that individual hermaphrodites usually produce more eggs than sperm, and that fecundity is limited by sperm production². Mutants that produced more sperm and fewer eggs left more offspring during their lifetime. The reason such mutants are selected against is that sperm are produced before eggs, so that the generation time for an individual that produces fewer sperm is shorter. In a growing population, which is where a reproducing *C. elegans* is likely to find itself, short generation time can be favoured over higher lifetime fecundity, and so the wild type can win against the more fecund mutants^{2,3}. Van Voorhies also uses mutants that are expected to change life-history schedules in predictable ways, and then sees if they do.

Sex differences

Biologists, who usually define males and females by the relative size of their gametes, believe that many other sex differences follow from this fundamental one. The argument generally runs along the following lines. If we assume that males and females have roughly the same amount of resources to devote to gamete production, then males will be able to produce more gametes than

females. Now make the further assumption that gametes are the only resources donated to the offspring, and it follows that female gametes will become a limiting resource for male reproduction. Males should compete to gain access for their sperm (or pollen) to eggs (or ovules)⁴. Such competition among males has led to the sexual selection of characters producing high male mating success, and hence the elaborate ornamentation and weaponry of the males of many species⁵.

Resources devoted to reproduction cannot be used for other vital processes, such as repair and maintenance, which are important for survival. Van Voorhies' experiments use decreases in rates of survival, or lifespan, to compare the costs of reproduction for both males and females. It is well established that such costs of reproduction can be important in practice⁶. If eggs are more expensive to produce than sperm, when a fixed number of eggs is added to a clutch we should expect to see a greater drop in survival for the female than for a male adding the same number of sperm. When hermaphrodites were mated to males (hermaphrodites will not mate with each other), the egg output went up two to threefold, yet lifespan in the mated hermaphrodites was no lower than that of unmated controls. In contrast, mating did reduce the lifespan of males and it is also known to increase their sperm production. Further evidence that the cost of mating was a consequence of making sperm, rather than the physical act of copulation, came from the finding that lifespan was increased relative to wildtype controls in both males and hermaphrodites that carried a mutant making them defective in spermatogenesis.

In the face of this evidence, the conclusion that each sperm costs more than each egg may seem hard to resist. However, males produce far more sperm (3,000+) than are needed to fertilize a hermaphrodite's eggs. Perhaps some of those sperm are metabolized into eggs after a hermaphrodite mates, thereby reducing the apparent cost of egg production. Nevertheless, high costs of spermatogenesis seem inescapable. Costs of reproduction for males are not new⁷, but the finding that spermatogenesis can so effectively curtail survival certainly is. On the basis of the data from the males alone, it could be argued that males are different from hermaphrodites in respects other than gamete production, and they also produce more sperm, and that is why males show such large reproductive costs.

Limiting factors

Sperm are qualitatively as well as quantitatively different from eggs, which may help explain why they are so costly to produce. Some support for this idea comes from a comparison of the rates of oogenesis versus spermatogenesis in *C. elegans*. Instead of the 1:500 ratio predicted by the size difference, sperm volume is produced at only 12.5 times the rate of egg volume. Sperm may contain a limiting nutrient that is present in larger quantities than in eggs, their synthesis may require more energy, or the rate of meiosis may be limiting. Whatever the explanation, *C. elegans* sperm are clearly limiting for female reproduction, contrary to the general expectation. Under such circumstances, hermaphrodites would be expected to compete for matings with males. Van Voorhies' unexplained finding that the act of mating itself appears to increase male lifespan would select further for a more than usually sex-mad male phenotype. Indeed, we are left wondering why selection has not succeeded in producing more males. □

Linda Partridge is in the Institute of Cell, Animal and Population Biology, Division of Biological Sciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK, and Paul H. Harvey is in the Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.

1. Van Voorhies, W. A. *Nature* **360**, 456–458 (1992).
2. Hodgkin, J. & Barnes, T. M. *Proc. R. Soc. B* **246**, 19–24 (1991).
3. Godfray, H. C. J. & Harvey, P. H. *Nature* **354**, 190–191 (1991).
4. Bateman, A. J. *Heredity* **2**, 349–368 (1946).
5. Trivers, R. L. in *Sexual Selection and the Descent of Man* (ed. Campbell, B.) 136–179 (Aldine, Chicago, 1972).
6. Bell, G. & Koufopanou, V. *Oxf. Surv. ev. Biol.* **3**, 83–131 (1986).
7. Partridge, L. & Farquhar, M. *Nature* **294**, 580–582 (1981).

A different kind of Universe

Giovanni F. Bignami

ON 17 November, γ -ray astronomy could be said truly to have come of age, as that was the day that the Compton γ -Ray Observatory, in orbit since April last year, completed the first-ever all-sky γ -ray survey. The significance of its mission became apparent at a meeting shortly before*, held suitably enough at Arthur Compton's old university, at which the results of the first 18 months were reviewed.

The Compton Observatory has a complement of four instruments covering the γ -ray energy spectrum from 30 keV to 30 GeV and capable of imaging the γ -ray sky, recording individual spectra and monitoring rapid variations and bursts from single sources. The meeting was more than a celebration of the satellite's capabilities; it was also a shop window for those looking to bid for time on the mission's third cycle.

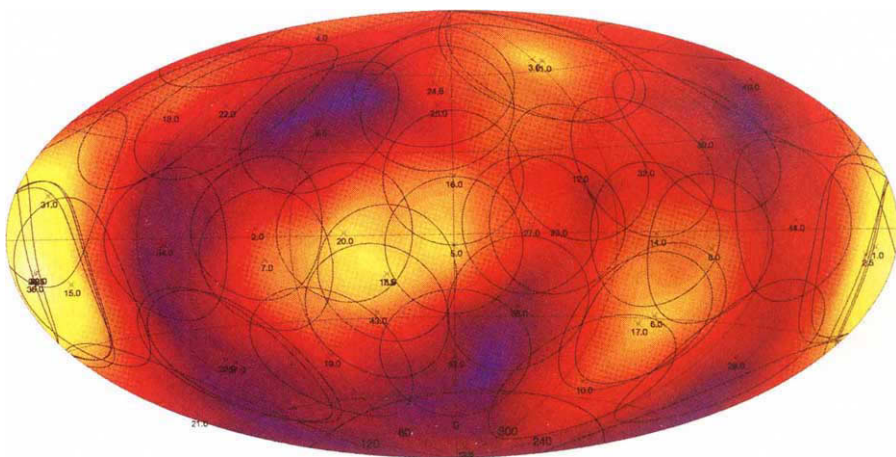
Among the Compton Observatory's early spectacular successes was the discovery of the brightest source of γ -rays, in the distant quasar 3C279 (see my News and Views article, *Nature* **355**, 299; 1992). A further 15 γ -ray quasars have since been discovered by the same instrument (EGRET, Energetic Gamma Ray Experiment Telescope), thanks to its sensitivity and ability to pinpoint sources. And COMPTEL (Compton Telescope) and OSSE (Oriented Scintillation Spectrometer Experiment) have seen several more. Although diverse in character and at a wide range of distances, the sources are all very powerful at radio wavelengths and are probably all characterized by a high degree of beaming along an axis. There were critics of γ -ray astronomy who did not believe it could give useful accuracy in pinpointing sources, but it is inconceivable that all the γ -ray quasars have been misidentified, especially as Compton's targets were carefully selected in advance. Instead, astrophysicists should now come to grips with the task of explaining how active galaxies come to radiate more energy at γ -ray wavelengths than over all the rest of the electromagnetic spectrum (even when the effects of beaming are accounted for).

The Compton Observatory has also dealt a severe blow to the idea that the

bulk of the cosmic ray background comes from beyond our Galaxy — that is from other galaxies. Using EGRET, the Compton team used a test proposed long ago by Vitaly Ginzburg, looking for γ -rays from the Magellanic clouds — the two small satellite galaxies in orbit about our own. The Small Magellanic Cloud, so far at least, appears to emit no γ -rays.

That brings much of γ -ray astronomy into the galactic domain. Several hundred pulsars are known, all recognized

Compton has also taken simultaneous observations of the spatial and spectral distributions of the 511-keV radiation produced by the annihilation of positrons on electrons (an indicator of an energetic astrophysical environment) and the 1.8-MeV radiation from radioactive aluminium-26. OSSE data show the annihilation radiation to be sharply peaked at the Galactic Centre, where its spectral shape is very narrow; there is, however, a significant spectral tail, indicative of the formation of the electron-positron 'atom', positronium. For both lines, whether the diffuse emission comes from genuinely diffuse sources or from unresolved point-like sources is



Painting the sky red: sky coverage by the Compton Observatory on its first survey, colour coded (blue to yellow) for exposure times in days (4–20). Fields of view of individual pointings are also shown.

through their distinctive radio pulsations, but despite theoretical predictions that these should produce copious γ -rays, only two (the Crab and Vela pulsars) had previously matched expectations. Their γ -ray emissions had been discovered in the 1970s by the SAS-2 astronomical satellite, and were subsequently confirmed by COS-B. With EGRET detections, that number has now more than doubled: pulsed γ -rays at energies above 100 MeV energy have been seen from Geminga — long known as a point source of γ -rays that defied explanation (see C. D. Bailyn's News and Views report in *Nature* **357**, 191; 1992) — and from the less notorious pulsar PSR1706–44.

Like the Crab and Vela examples, PSR1706–44 is a *bona fide* radio pulsar, but its γ -ray phase pattern is somewhat different. Geminga, it now appears, is our closest pulsar, but its geometry seems to have prevented us from seeing any radio signal from it. At lower energies, γ -ray pulsars are apparently harder to find, nevertheless COMPTEL and BATSE (Burst and Transient Source Experiment) have identified pulsed γ -rays below 10 MeV from Vela, as well as sub-MeV γ -rays from PSR1509–58.

not clear. Distinct sources would be expected for the 1.8-MeV line, as this is the product of the magnesium-aluminium nucleosynthesis cycle which occurs in supernovae, nova outbursts, Wolf-Rayet stars and so on.

The most celebrated of all the results from Compton, touched on many times in these pages, concerns γ -ray bursts — brief outbursts of γ -radiation, typically not repeated from any particular source — whose origin remains a complete mystery. In the absence of any better means of resolving the debate, the conference organizers resorted to the unorthodox but democratic method of a show of hands. The galactic and extragalactic parties polled even support; sources in the Solar System gathered a few votes. But there was a substantial rump in support of an agnostic approach — perhaps the most sincere position given the self-contradictory character of the Compton data. □

Giovanni F. Bignami is in the Istituto di Fisica Cosmica, Via E. Bassini 15, 20133 Milano, Italy.

* Compton Symposium, Washington University, St Louis, 15–17 October 1992.

Correction

In the article dealing with the mechanism by which β -lactamase nullifies the effects of penicillin (*Nature* **359**, 674; 1992), it was stated that the antibiotic ampicillin (the correct spelling), is resistant to β -lactamase; it is in fact sensitive. In the same article glutamine 166 should have read glutamate 166.

Another route to a vaccine?

F. E. G. Cox

OVER the past few years, the search for a vaccine against malaria has shifted away from the infective sporozoite injected by the mosquito to the next phase of the life-cycle, the pre-erythrocytic stage in the liver (see box). A major problem, however, is how a protective mechanism against this stage might work. Now an important clue is reported on page 434 of this issue¹, which stems from earlier observations by Adrian Hill and his colleagues that there is an association between the HLA class I antigen HLA-B53 and resistance to severe malaria².

Their original hypothesis was that the HLA-B53 molecule might act as a binding site for a malaria antigen expressed on the surface of hepatocytes occupied by the parasites, and that this antigen might be a target for cytotoxic T lymphocytes (CTL). Using a series of 60 synthetic 8- 9- and 10-amino-acid peptides that matched the HLA-B53 molecule, and relating these to known sequences of sporozoite and liver-stage malaria antigens, Hill and his co-workers have pinpointed one likely candidate, a

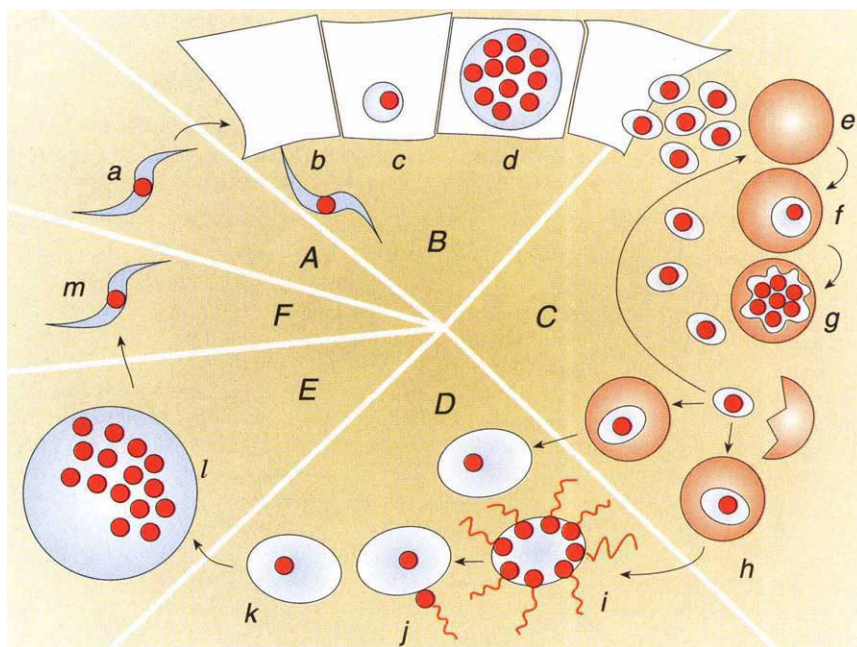
9-amino-acid epitope, 1s6, from a specific liver-stage antigen LSA-1 (ref. 3). Interestingly, none of the sporozoite antigens is incriminated. The hope is that the 1s6 epitope could be a component of a vaccine that might induce a specific CTL response against the early stages in the liver and thereby confer some degree of immunity against the parasite. If hope turns into reality, this would be the first application of 'reverse immunogenetics' in the quest for epitopes suitable for vaccine design.

After many years of obscurity, the liver stage of the malaria life-cycle now seems to be a good target for immunological attack. The reasons for ignoring this stage until recently have been the excessive attention paid to antibody-mediated responses to the sporozoite. In addition, there has been an unwillingness to accept the possibility that CTL could kill a parasite-infected cell, although it has been known for some time that the major defence against the cattle protozoa *Theileria parva* and *T. annulata* is not only CTL-dependent and restricted by the major histocompatibil-

ity complex, but also that there are very effective vaccines based on the stimulation of this cytotoxic response⁴. Until now, however, the evidence for CTL involvement in malaria has been based largely on animal models and on sporozoite antigens either expressed during the early stages in the liver or left on the hepatocyte surface after invasion⁵. In humans, CTL responses to an immunogenic sporozoite antigen alone are not adequate to protect against malaria; furthermore, the protective epitopes contain polymorphic residues, suggesting that a protective immune response directed against one strain of the parasite might not be effective against another⁶. The advantage of the 1s6 epitope is that it seems to be conserved, at least in the Gambia¹, and is thus a potentially more useful molecule.

So far so good, but the significance of an association between HLA-B53 and protection against severe malaria has been questioned. The criteria used for defining severe malaria include cerebral involvement, hypoglycaemia and anaemia; the pathology of malaria may in part be due to a combination of immune responses⁷. Neither the proponents nor the opponents of the HLA-B53 protective association hypothesis have considered the real problem, which

Life cycle of a malaria parasite showing the stages that might act as targets for vaccine-induced immunity. The cycle begins when infective stages, sporozoites (a), are injected directly into a blood vessel (A) by a mosquito. The sporozoite is an obvious target for antibody-mediated attack, but the success of vaccines against this stage has been disappointing. Sporozoites then enter liver hepatocytes (B) where they divide to produce up to 10,000 merozoites. Possible targets include blocking sporozoite entry into the liver by antibodies (b), or killing early developmental stages in the liver by cell-mediated mechanisms (c) as described above. There is no evidence of any immune killing of the late-liver stage (d). Merozoites released into the bloodstream (C) invade red blood cells (e) where further division then occurs, resulting in the production of 8–16 merozoites which repeat the cycle. A number of antibodies inhibit invasion of red cells (e) or the development of young (f) or mature (g) intraerythrocytic stages, but attempts to produce vaccines against these stages have met with mixed success. The bloodstream forms are also responsible for the pathology associated with malaria, part of which is immunologically mediated. Vaccines that neutralize toxic substances released by the parasites are being considered. Sexual stages (h) are



also produced in the blood and are taken up by a mosquito when it feeds (D). Within the mosquito gut, a male gametocyte (i) produces gametes that fertilize a female gamete (j) to produce a zygote (k). Antibodies, induced either naturally or artificially, inhibit all these sexual stages and vaccines against these stages have been used experimentally for blocking transmission. The zygote then forms an oocyst (l) in the gut wall of the mosquito (E), where another massive phase of multiplication results in the production of sporozoites (m) that enter the salivary glands (F) and are injected into the human host. There is no evidence of any immunity to these mosquito stages.

F. E. G. C.

How to give a galaxy a black eye

THE 'Evil Eye' or 'Black Eye' galaxy (NGC4826) owes its ominous form and name to a remarkable and complex dynamical structure, R. Braun, R. A. M. Walterbos and R. C. Kennicutt Jr report on page 442 of this issue. Like other spiral galaxies, NGC4826 contains a disk of stars which all rotate in the same direction about the centre of the galaxy. However, the band of gas and dust that gives NGC4826 its Evil Eye rotates in the opposite direction to the stars, quite unlike the gas motion in normal galaxies. Encounters between the rotating and counter-rotating gas clouds would quickly suppress the counter-rotating component within one galactic revolution (100 million years), so that the Evil Eye is clearly in a transient state of affairs.

Most probably, the Evil Eye represents the aftermath of a recent collision between NGC4826 and a smaller, gas-rich galaxy. The smaller galaxy would have collided with NGC4826 on an orbit that was retrograde with respect to the stars in the spiral, leaving gas and dust spread out in a counter-rotating disk. We know that the galaxy that collided must have been considerably smaller than NGC4826 because the disk of NGC4826 is still present; disks are very fragile dynamical systems and are easily disturbed and destroyed by tidal encounters with galaxies of comparable mass.

Even minor encounters with galaxies as small as 5–10 per cent of the

disk's mass can thicken the disk by a factor of two or more. Spiral galaxies, with their fragile stellar disks, have been nurtured in parts of the Universe that are quiet backwaters, free from significant encounters. The sensitivity of the disks to even minor encounters implies that for at least the past 5–10 billion years the immediate environment of spirals has been free from small objects on orbits that would penetrate the disk.

If cosmological 'N-body' models of the growth of galaxies are correct, then all galaxies grew by a process of

agglomeration. This agglomeration must have ceased sufficiently long ago for spirals to have since formed their disks. Unusual recent events like the collision that gave rise to the Evil Eye afford us an ideal opportunity to study the types of encounters that were once common when the large galaxies were first assembled.

P. J. Quinn

P. J. Quinn is at Mount Stromlo and Siding Spring observatories, Australian National University, Private Bag, Weston Creek, ACT 2611, Australia.



US Naval Observatory/Hansen Planetarium

is that immunity to malaria is largely stage-specific: a vaccine against the liver stage might prevent the infection from developing, but the link with severe malaria is not obvious, given that by definition this is associated with the blood stages. Nevertheless, the systematic approach adopted by Hill and his colleagues has identified a potentially useful antigen and it must now be considered as a candidate for inclusion in a cocktail vaccine.

This is a good time to take stock of the way in which the development of a malaria vaccine is likely to go. The dominant repeat region of the sporozoite protein (CSP) seems to have had its day, but the possibility of hindering the invasion of host hepatocytes by immunizing against the sporozoite antigens involved in cell recognition⁸ is a possibility, although several epitopes will probably have to be neutralized to prevent invasion entirely. Despite the fact that few antigens specific to the liver stage have yet been recognized, the liver stage is

now an obvious target, and interest in these molecules will increase when other groups take up the approach of identifying antigens targeted by CTL. The blood stages still remain the principal target and, in this context, the recent results of Manuel Patarroyo's synthetic vaccine trials in Colombia are interesting. Out of 185 service personnel treated with a vaccine that elicited an antibody-mediated immune response, only 2 became infected after duty in a malarious zone, compared with 9 out of 214 placebo controls⁹. Although it is difficult to justify the claim of 60–80 per cent efficacy on these figures, they do suggest that the vaccine could be having some effect.

Perhaps this is also the time to look at the successes, both theoretical and practical, already achieved and to view the future realistically. There are direct comparisons in other fields: for example in infections with human immunodeficiency virus, some feel that the prospect of a vaccine is improbable¹⁰, whereas others

accept that although there is a long way to go, much can be learned from 'first generation' vaccines¹¹. What happens in the field is more important than what happens in the laboratory, and obtaining clues from HLA associations is a good way to begin. □

F. E. G. Cox is in the Division of Life Sciences, King's College London, Campden Hill Road, London W8 7AH, UK.

- Hill, A. V. S. *et al.* *Nature* **360**, 434–439 (1992).
- Hill, A. V. S. *et al.* *Nature* **352**, 595–600 (1991).
- Zhu, J. & Hollingdale, M. *Molec. biochem. Parasitol.* **48**, 223–226 (1991).
- Morrison, W. I. *et al.* *Parasite Immun.* **9**, 563–578 (1987).
- World Health Organization *Bull. Wild Hlth Org.* **68** (suppl.), 1–196 (1990).
- Malik, A., Egan, J. E., Houghten, R. A., Sadoff, J. C. & Hoffman, R. *et al.* *Vaccine* **10**, 179–184 (1992).
- Carter, R., Schofield, L. & Mendis, K. *Parasitol. Today* **8**, 41–42 (1992).
- Cerami, C. *et al.* *Cell* **70**, 1021–1033 (1992).
- Amador, R. *et al.* *Vaccine* **10**, 179–184 (1992).
- Sabin, A. B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8852–8855 (1992).
- Ada, G., Blanden, B. & Mullbacher, A. *Nature* **359**, 572 (1992).

Light in the dark

Elliot M. Meyerowitz

WRITING in *Cell*¹, X.-W. Deng and colleagues describe the cloning of one of a particular class of genes concerned in the control of a plant's response to light. That achievement, and the authors' related observations, will help in the endeavour to understand plant phototransduction. The paper is also the latest in a series reporting the association of familiar functional modules of regulatory proteins in unfamiliar combinations; this is a line of plant research that is turning in a number of surprises and will be of interest to a much wider community.

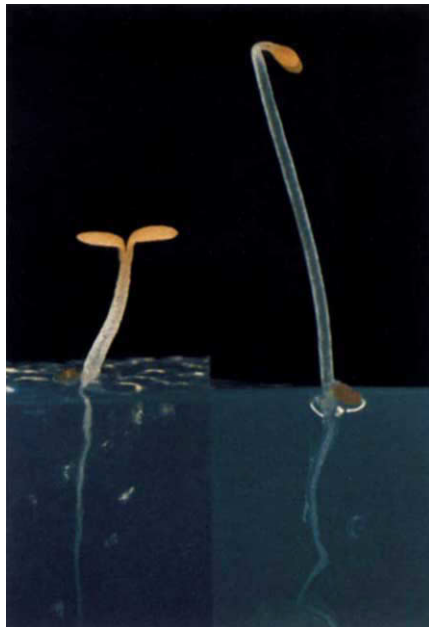
Rather than using neurons and muscles to respond to the environment, plants have evolved plastic forms of development that use cell division, elongation and differentiation for the purpose. Among the environmental stimuli with the most dramatic effects is light. A plant grown in the dark comes to have elongated stems, small leaves and undifferentiated chloroplasts (it is white). The long stems are thought to be an adaptive response, an attempt by a shaded or underground plant to move its growing point to a sunnier environment.

Several laboratories have been attempting to understand this type of light control of development by looking for mutants in which such control is absent, causing plants to behave in the light as if they were in the dark or vice versa. Both types of mutants have been found. An example of a plant that acts (in one way) as if it were in the dark, regardless of its illumination, is the *HY3* mutant of *Arabidopsis thaliana*². This mutant germinates normally, but begins growth with an overly long stem and does not become fully green. Molecular evidence shows that the *HY3* gene codes for the apoprotein of one of the *Arabidopsis* phytochromes^{3,4}, the photoreceptors for long-wavelength light.

A more puzzling class of mutants is that in which dark-grown plants behave as if they were in the light⁵⁻⁷. The existence of recessive mutants of this type implies that the effect of light is to inactivate repressors of photomorphogenesis. If so, cloning these genes should reveal some of the mechanisms by which phytochromes (and the other plant photoreceptors) act.

The first gene in this class has now been cloned by Deng *et al.*, from *Arabidopsis*¹. The gene is called *COP1* (for constitutively photomorphogenic); the recessive mutant phenotype is that seedlings grown in the dark (see figure) have a morphology and form of cellular differentiation similar to that of plants grown in the light. Intriguingly, *COP1*

codes for a protein with two familiar motifs: it has a zinc-finger region near its amino terminus, and four copies of the WD-40 repeat towards its carboxy-terminal end. The WD-40 repeat is of unknown function, but is found in a variety of regulatory proteins^{8,9}, including heterotrimeric G protein β -subunits ($G\beta$) and the yeast transcriptional repressor TUP1.



A four-day-old dark-grown *COP1* mutant seedling of *Arabidopsis thaliana* (left) in comparison with a dark-grown wild-type seedling of the same age (right). (Courtesy of X.-W. Deng.)

This finding¹ does not reveal the mechanism of plant phototransduction, but it does raise several interesting possibilities. The WD-40 proteins emphasized by Deng *et al.* are TUP1 and $G\beta$. TUP1 acts as a transcriptional repressor in association with another protein, SSN6 (ref. 10). These proteins are thought to be recruited to their target promoters by additional, sequence-specific DNA-binding proteins. This is certainly consistent with the proposed function of *COP1*, that it acts as a repressor of the photomorphogenic pathway. The similarity of *COP1* to the β -subunit of heterotrimeric G proteins is also consistent with evidence that light responses mediated by phytochrome and blue light may act through G proteins^{11,12}, as do rhodopsin-mediated light responses in animals.

Quite a different possibility is that the *COP1* protein is a regulated component of a specific RNA-splicing pathway. Yeast PRP4, a part of the U4/U6 small

nuclear ribonucleoprotein, is a WD-40 protein^{13,14}; and PRP11 (RNA11), a protein that is part of the spliceosome, has a zinc-finger region¹⁵. Both proteins are required for RNA splicing, PRP4 early in spliceosome assembly, and PRP11 as a spliceosome component. So these motifs are known to act together (though on different protein molecules) in processing pre-messenger RNA. Could they function together, on the same protein molecule, to regulate light-specific RNA-splicing patterns in plants?

The finding of hitherto unknown associations of two familiar protein domains is becoming a regular result of the cloning of plant genes. Among the other examples are the *Arabidopsis* homeobox leucine-zipper proteins^{16,17}, and the calcium-dependent protein kinase of soybean that contains a calmodulin motif¹⁸. Whether similar proteins to these exist in animals is still unknown. One possibility is that they do. Another is that plants and animals have similar functional protein motifs, inherited as part of their prokaryotic or unicellular eukaryotic legacy, but that they use these motifs in different combinations.

One of the main issues raised by the parallel study of plant and animal development is the extent to which developmental processes in the two kingdoms resemble each other. Each kingdom, as far as we know, evolved multicellular development independently. Is there only one way to do it? Or are the solutions to common developmental problems based on commonly inherited and ancient protein domains, used in very different ways? These are profound questions, the answers to which will eventually come from continued cloning and sequencing of developmentally important genes such as *COP1*. □

Elliot M. Meyerowitz is in the Division of Biology, California Institute of Technology, Pasadena, California 91125, USA.

- Deng, X.-W. *et al.* *Cell* **71**, 791–801 (1992).
- Koornneef, M. *et al.* *Z. PflanzenPhysiol.* **100**, 147–160 (1980).
- Nagatani, A. *et al.* *Pl. Cell Physiol.*, Tokyo **32**, 1119–1122 (1991).
- Somers, D. E. *et al.* *Plant Cell* **3**, 1263–1274 (1991).
- Chory, J. *et al.* *Cell* **58**, 991–999 (1989).
- Chory, J. & Poto, C. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 8776–8780 (1990).
- Deng, X.-W. *et al.* *Genes Dev.* **5**, 1172–1182 (1991).
- Fong, H. K. W. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **83**, 2162–2166 (1986).
- van der Voorn, L. & Ploegh, H. L. *FEBS Lett.* **307**, 131–134 (1992).
- Keleher, C. A. *et al.* *Cell* **68**, 709–719 (1992).
- Bossen, M. E. *et al.* *Physiologia Pl.* **80**, 55–62 (1990).
- Warpeha, K. M. F. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **88**, 8925–8929 (1991).
- Bjorn, S. P. *et al.* *Molec. cell. Biol.* **9**, 3698–3709 (1989).
- Banroques, J. & Abelson, J. N. *Molec. cell. Biol.* **9**, 3710–3719 (1989).
- Chang, T.-H. *et al.* *Molec. cell. Biol.* **8**, 2379–2393 (1988).
- Ruberti, I. *et al.* *EMBO J.* **10**, 1787–1791 (1991).
- Schena, M. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **89**, 3894–3898 (1992).
- Harper, J. F. *et al.* *Science* **252**, 951–954 (1991).

Gene puffs

GENE therapists exercise their skills with high-tech tools borrowed from viruses to correct faulty genes in cells. A different approach to the same end is described by R. Stribling *et al.*, who used cationic liposomes to wrap up a prokaryotic gene for direct delivery by aerosol into the lungs of mice (*Proc. natn. Acad. Sci. U.S.A.* **89**, 11277–11281; 1992). The bacterial protein went on being expressed in all the conducting airways of the lung for more than three weeks after the gene encoding it was inhaled, apparently without damage to the mouse. Although any effects of repeatedly sniffing these high-fat DNA particles still need to be determined, the authors are optimistic that their technique may one day be used to help sufferers of cystic fibrosis.

Censor's triumph

Cosmic censorship, the hypothesis formulated to allow general relativists to sleep at night, has it that any gravitational singularity must always be concealed behind an event horizon. Whether this is invariably true is an unanswered question, and some relativists, eager to disturb their colleagues, have tried to construct 'naked' singularities — unsuccessfully so far. A. Abrahams and C. Evans (*Phys. Rev. D* **46**, R4117–R4121; 1992) have also tried to evade the cosmic censor. By careful arrangement of initial conditions, they have numerically calculated the evolution of an imploding gravitational wave with axisymmetric (quadrupole) symmetry. The energy in the wave converges on a point, increasing the local density without limit so that a singularity forms. But, as always, an event horizon forms first, and the singularity is hidden. What Abrahams and Evans have found, instead, is an extremely difficult way to make a black hole.

Waste management

Do pea aphids, *Acyrtosiphon pisum*, get by with a little help from their symbiotic bacterial friends in recycling aphid waste ammonia to essential amino acids? Yes (or probably yes) conclude L. F. Whitehead *et al.* (*Proc. R. Soc. B* **250**, 115–117; 1992). The authors took two approaches. Bacteria isolated from the aphid can accumulate methylamine, an analogue of ammonia; and if in some unhappy aphids the bacteria are disrupted by antibiotics, the ammonia content in their excreta rises 3–4 times. Whitehead *et al.* tie these results into work showing that bacteria release amino acids essential for the aphids. But the conclusion remains tentative: it is not entirely certain that methylamine and ammonia share the same transport system in bacteria, or that something other than the bacteria is not perturbed by the antibiotics.

A tale of two families

Susan G. Amara

MAJOR advances in the cloning of neurotransmitter transporters over the past three years make this the best of times to examine how these high-affinity reuptake systems terminate synaptic transmission. The notable exceptions to this cloning onslaught have been the eagerly anticipated DNA sequences encoding the glutamate transporters, the glial and neuronal uptake systems that sequester glutamate released at the most abundant class of excitatory synapses. The reason for this curious omission is now evident from three papers, one in the *Proceedings of the National Academy of Sciences*, and two appearing on pages 464 and 467 of this issue: glutamate transport is accomplished by a multigene family distinct from the gene family that includes all other neurotransmitter transporters isolated so far^{1–3}.

The molecular characterization of neurotransmitter carriers began with the purification, amino-acid sequencing and cloning of a γ -aminobutyric acid (GABA) transporter, and was consolidated when the expression cloning of the noradrenaline transporter demonstrated that a transporter gene family exists. Since then, a growing list of neurotransmitter transporters have been isolated by methods that rely on the sequence conservation within the 12 transmembrane structural motifs of this gene family⁴. Members of the family, which includes transporters for the biogenic amines, GABA, glycine, choline, proline and taurine, all seem to link substrate influx to the cotransport of Na^+ and Cl^- ions across the plasma membrane. Glutamate transport, however, has appeared mechanistically distinct because amino-acid influx is coupled to the cotransport of Na^+ and the countertransport of K^+ , with no dependence on Cl^- . Such functional distinctions are now explained by the demonstration that glutamate transporters represent a very different structural class of amino-acid carriers.

Three distinct complementary DNAs encoding structurally related excitatory amino-acid transporters have now been isolated by returning to methods that do not rely on family relationships. Serendipity led Storck *et al.*¹ to a glutamate/aspartate transporter cDNA, referred to as GLAST 1, which was identified using oligonucleotide probes based on the sequence of a protein that co-purified with a ceramide galactosyl transferase. The RNA encoding the transporter is specifically expressed in the brain, and although its precise cellular location has not been determined its diffuse distribution and properties may be more con-

sistent with those of a glial glutamate transporter. Pines *et al.*² purified a protein with glutamate transport activity from rat brain and used the protein to obtain antibodies for screening a bacteriophage cDNA expression library. The resulting cDNA clone, referred to as GLT-1, detects an RNA species in rat brain, but not in any peripheral tissues tested, and in a separate report the authors provide evidence that, like GLAST 1, the GLT-1 gene product is expressed predominantly in glial cells⁵.

Using a different approach, Kanai and Hediger³ screened a plasmid cDNA library for expression of high-affinity uptake activity in *Xenopus* oocytes to obtain a cDNA which they term EAAC1. *In situ* hybridization suggests that the expression of EAAC1 within the central nervous system is largely neuronal, and it includes expression within the hotbed of glutamatergic synapse research, the pyramidal cells of the hippocampus. Neurobiologists may find the distribution of the EAAC1 gene product rather surprising, because although the high-affinity transporter is expressed abundantly in the central nervous system, it is also found in several peripheral tissues and was actually identified in a cDNA library from rabbit small intestine.

GLAST 1, GLT-1 and EAAC1 are very similar in their amino-acid sequences (~50% identical) and in their proposed structural characteristics, establishing these genes as members of a new family. Surprisingly, database searches for related sequences by all three groups found significant homology with the proton-coupled glutamate transporter *glt-P* of *Escherichia coli*⁶, as well as several other prokaryotic glutamate and dicarboxylate transporters. Particularly striking is the conservation of the seven-amino-acid sequence AAIFIAQ found both in the *E. coli* and mammalian proteins, which suggests a rather longstanding familial relationship. The proposed membrane topology of these glutamate transporters is somewhat ambiguous, given the subjective application of structural algorithms. There is general agreement on the location of the first six transmembrane domains in the three transporters, but the laboratories differ on the total number of membrane-spanning segments, proposing 6, 8 or 10. In the light of such controversy, perhaps it is better to distinguish the two plasma membrane transporter gene families not by the number of transmembrane domains (12 versus 6–10), but by their ionic depend-

ence: either Na^+/Cl^- or Na^+/K^+ .

A report by Bouvier *et al.* on page 471 of this issue⁷ adds an interesting new twist to our understanding of the distinct ion dependence of excitatory amino-acid transport: in addition to Na^+ and K^+ , hydroxyl anions (OH^-) seem to be carried by the transporter. The new report provides direct evidence that, in salamander retinal glial cells, the transport of glutamate is accompanied by increases in extracellular pH and decreases in intracellular pH which are caused by the outward transport of OH^- anions. These findings clarify previous studies of aspartate transport in synaptosomes, where alkalization of the external medium associated with transport had first been noted⁸. The new results have led to a proposed stoichiometry for glutamate transport in which the uptake of one glutamate anion and two Na^+ ions is coupled to the countertransport of one K^+ ion and one OH^- anion.

The termination of synaptic transmission is a function that glutamate transporters share with the Na^+/Cl^- -dependent family of neurotransmitter transporters. However, glutamate transporters have an additional important role in the central nervous system: preventing or limiting excitotoxic cell death. Excessive local concentrations of glutamate have been implicated in selective neuronal destruction in epilepsy, stroke and other anoxic/ischaemic states, as well as in a variety of idiopathic and hereditary neurodegenerative diseases such as amyotrophic lateral sclerosis and Huntington's disease. Glial glutamate transporters in particular may have a critical role in maintaining glutamate concentrations below neurotoxic levels.

Because of the unique ionic dependence of glutamate transport, significant changes in the extracellular and/or intracellular concentrations of Na^+ or K^+ can have dramatic effects on transporter function. Three conditions known to occur during ischaemia or anoxia, namely increased extracellular K^+ , decreased extracellular Na^+ and a depolarized membrane potential, can severely compromise the rate of transport and even favour reversal of the transport process. Glutamate transporters are

electrogenic and so electrophysiological approaches have been used to show that net efflux of glutamate takes place under these conditions and may contribute to the neurotoxic concentrations of extracellular glutamate^{9,10}.

Together with recent breakthroughs in the cloning of glutamate-gated ion channels and receptors, the identification of these glutamate carriers provides us with structural information on the major components of the synaptic signalling apparatus. In this context it should

now be possible to move on to the exploration of the molecular mechanisms of transport and the role of specific transporters in excitatory amino-acid neurotransmission. As is often the case with new families, more members and more than a few surprises are likely to be encountered along the way. □

Susan G. Amara is at the Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, Oregon 97201, USA.

GEOPHYSICS

Preserving a sense of direction

S. R. Dickman

WITH the Earth's continental and oceanic plates constantly shifting, its magnetic field reversing, and its rotation axis still reeling from the geologically recent glaciations, should we expect the rotation axis to have been wildly unstable throughout geological history? Apparently not, for reasons laid out by Spada *et al.* on page 452 of this issue¹.

The exotic possibility that the Earth has undergone major episodes of 'true polar wander' — so-called because of the apparent displacement of the pole over the Earth's surface, as the globe turns over relative to space — was first raised well over a century ago with the discovery that currently tropical regions had long ago been glaciated, and tropical plants had at the same time thrived in now polar climates. Solid theoretical foundations of the mechanisms for exciting true polar wander (TPW) were laid out by George Darwin (son of Charles) and others. The conceptual peak of the theory was perhaps reached by Thomas Gold², who described in 1955 the excitation of TPW on a plastically deformable Earth by the most insignificant of effects: changes in the globe's moment of inertia due to the crawling of a tiny beetle over the Earth's surface.

A decade after Gold's article, however, it became clear that the original observations could be better explained within the developing paradigm of plate tectonics, as a consequence of the continental drift of glaciated lands into tropical latitudes and the converse. The consensus, in the words of John Verhoogen³, became "continents certainly move while poles possibly do not".

In the intervening years, a wide variety of evidence has kept the possibility of long-term TPW alive. Furthermore, the present-day motion of the rotation pole towards Hudson's Bay has been recognized as a continuing TPW and identified, ironically, as a consequence of global mass redistribution following the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Shifting ground: the Earth as seen by Meteosat. (European Space Agency/Science Photo Library.)

geologically recent episodes of glaciation and deglaciation. Such mass redistribution includes the viscoelastic migration of the Earth's equatorial bulge, which has attempted during the past few million years to maintain its alignment perpendicular to the rotation axis (in the fashion of Gold's beetle-induced TPW) following episodic perturbations of the latter by surficial loads. Given the success of such models of the viscoelastic Earth, it is natural to wonder how the Earth's evident ability to undergo geologically short-term rapid TPW might be reconciled with times of relative quiescence despite the innumerable other sources of excitation that have occurred throughout geological time.

Spada *et al.*¹ address that question quantitatively, applying to the problem of long-term TPW the same geophysically sophisticated models of the Earth that successfully explained the recent shift of the pole. In what amounts to a modernization of Gold's beetle (or its generalization to multiple beetles by Goldreich and Toomre⁴), the authors

1. Storck, T., Schulte, S., Hofmann, K. & Stoffel, W. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10955–10959 (1992).
2. Pines, G. *et al. Nature* **360**, 464–467 (1992).
3. Kanai, Y. & Hediger, M. A. *Nature* **360**, 467–471 (1992).
4. Amara, S. G. & Kuhar, M. J. A. *Rev. Neurosci.* **16**, 73–93 (1993).
5. Danbolt, N. C., Storm-Mathisen, J. & Kanner, B. I. *Neuroscience* (in the press).
6. Tolner, B., Poolman, B., Wallace, B. & Konings, W. N. *J. Bact.* **174**, 2391–2393 (1992).
7. Bouvier, M., Szatkowski, M., Amato, A. & Attwell, D. *Nature* **360**, 471–474 (1992).
8. Erecinska, M., Wantorski, D. & Wilson, D. F. *J. biol. Chem.* **258**, 9069–9077 (1983).
9. Nicholls, D. & Attwell, D. *Trends pharmac. Sci.* **11**, 462–468 (1990).
10. Szatkowski, M., Barbour, B. & Attwell, D. *Nature* **348**, 443–446 (1990).

MEASURES OF TRUE POLAR WANDER

Data set . . .	implies ancient motion of . . .	relative to . . .	assuming that . . .
Palaeoclimatic	The Earth's surface	► Climate zones ► Rotation axis	— Rotation axis has determined climate zones
Palaeomagnetic	Lithospheric plate on which site is located	► Palaeomagnetic dipole axis ► Rotation axis	— Rotation axis coincides with average palaeodipole axis
Palaeomagnetic, common to all plates	Entire lithosphere	► Palaeomagnetic dipole axis ► Rotation axis	— Rotation axis coincides with average palaeodipole axis
Hotspot tracks	Lithospheric plate on which site is located	► Underlying mantle ► The Earth	— Hotspots are fixed with respect to deep mantle
Hotspot tracks and palaeomagnetic	Rotation axis	► The Earth	Preceding assumptions

bring together the dynamics of TPW and those of convection, the underlying flow of the Earth's mantle which drives continental drift.

Mantle convection reflects the dynamics of the Earth as a heat engine: hot, relatively light mantle material wells up from the deep interior, spreading out beneath the rigid, plate-like top layer of the Earth and carrying sections of the rigid lithospheric layer (and continents embedded within them) as it does so. When the laterally flowing mantle material, and overlying oceanic plates, have cooled sufficiently, they are dense enough to sink back down — convective 'beetles' of slight excess mass diving back into the deep mantle.

Spada *et al.* model the convective return flow, which begins at lithospheric subduction zones in the uppermost mantle, as a sequence of randomly located downward-moving beetles, generated at a fixed time interval that could be either 2 or 10 million years. The Earth's total mass is conserved at each instant as the mass anomalies sink through the mantle, and a nonlinear version of the angular-momentum equations is used so that large-amplitude displacements can be accurately computed. The authors' conclusion is robust: regardless of the details of 'beetle activity', the typical rate of true polar wander should be very large (5° – 10° per million years) if the mantle viscosity is uniform. Only if the lower mantle's viscosity is at least 10 times greater than the upper mantle's could the rate of TPW be as small as 0.5° per million years; a viscously thickened lower-mantle fluid would slow both the rate of beetle descent and the rate of bulge migration.

Although the net displacement of the

actual pole is inferred to have been negligible during the past 50 million years, a combination of palaeomagnetic and hotspot data⁵ (see table) suggests that further back — during the early Tertiary/late Cretaceous epochs — the Earth apparently underwent a period of comparatively rapid TPW, at rates amounting to 0.5° per million years. In both cases, the rate of TPW falls into the theoretical category of Spada *et al.*, requiring a thickened lower mantle. Whether the new analysis can be applied to the real Earth may depend on the extent to which actual convective flow — during which density variations, and so mass anomalies, are somewhat gradual and continual — can be represented by discretely sinking randomly generated beetles. Random beetles may also promote more of a destructive-interference cancellation of TPW effects (see Fig. 2 of Spada *et al.*) than would a realistic, nonrandom distribution of subduction mass anomalies. But if this is true, the implications are even more robust. Thus it appears that, even with innumerable sources of excitation throughout the Earth's history, the rheological structure of the lower mantle has dampened the Earth's true polar wander response. □

S. R. Dickman is in the Department of Geological Sciences, State University of New York, Binghamton, New York 13902, USA.

1. Spada, G., Ricard, Y. & Sabadini, R. *Nature* **360**, 452–454 (1992).
2. Gold, T. *Nature* **175**, 526–529 (1955).
3. Verhoogen, J. *et al.* *The Earth* (Holt, Rinehart and Winston, New York, 1974).
4. Goldreich, P. & Toomre, A. J. *geophys. Res.* **74**, 2555–2567 (1969).
5. Besse, J. & Courtillot, V. J. *geophys. Res.* **96**, 4029–4050 (1991).

DAEDALUS

Forced food

COOKING has three main functions. It has to degrade toxins and kill bacteria in the food; it has to render it digestible, mainly by breaking or weakening cell walls to make their contents accessible; and it has to develop an appealing flavour. With the development of the microwave oven, this last purpose is fast falling into abeyance. Traditional cooking imposes a steep temperature gradient across an item of food. This generates a wealth of intriguing tastes and smells, ranging from those of extreme pyrolytic degradation on the outside to the savour of almost unchanged ingredients in the middle. By contrast, uniform microwave heating produces a tasteless, pallid, warmed-up product whose flavour has to be supplied by chemical additives.

So Daedalus is developing an entirely new cooking technology. He recalls a clever trick which molecular biologists use to extract proteins from *Escherichia coli*. To avoid the heat and violence of conventional grinding or ultrasonic agitation, they simply burst the organisms by explosive decompression. DREADCO chefs are now adapting this technology to food preparation.

Their novel 'pressure cooker' simply pressurizes the food with cheap, safe carbon dioxide, a gas which can even be taken super-critical at room temperature. It is then such a good solvent that it enters, softens and plasticizes the toughest tissues. When the pressure is released, it expands back to gas. Every cell in the food swells up and bursts. All contaminating bacteria are killed, all cell contents are released, all coherent structures are disrupted by the violently expanding gas.

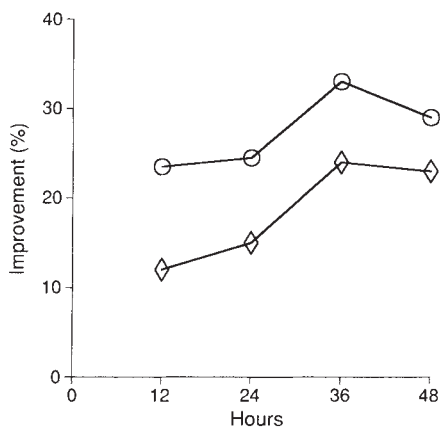
This wonderful process works on the most dubious raw material. Offal and vegetable waste which cannot even be made into sausages or dog food, even general biological debris like grass cuttings and leather fragments, can be safely 'popped' by the DREADCO process. It doesn't matter if it is contaminated by bacteria, or even rats — they are popped too. The whole awful mess is broken down into an open, fibrous powder, sterile but nutritive, and rather like damp sawdust; DREADCO's 'Popped Pabulum'. The consumer will never know what he is eating; and it won't matter.

Fast food will thus attain its ultimate development. All potential food, however revolting, will be instantly recoverable. Popped Pabulum will multiply the food resources of the world many times. Suitable synthetic flavour additives could even make it quite palatable. Toxins and viruses in the raw material will, however, survive unaltered. They will be destroyed in the subsequent microwaving. David Jones

Predicting hurricane tracks

SIR — Predicting the paths of hurricanes has been extremely difficult. Over the past 30 years or so, the reduction in the 24-hour forecast track error has averaged only 0.5% per annum. This is largely a consequence of hurricanes spending almost all of their lives over the data-sparse oceans. Dynamical models of hurricanes have also been blamed, for being too simple. Indeed, such models have exhibited less forecast skill than statistically based methods, particularly in the first 24 or even 36 hours.

A superior data set has been collected in the hurricane-rich south China Sea



Hurricane track predictions. Percentage improvement in (reduction of) mean forecast error to 48 hours ahead, relative to climatology persistence (CLIPER). Diamonds, standard initialization; circles, initialization by generalized inversion. By convention the 7% difference at 48 hours, for example, is scaled with respect to the 70% residual, yielding a '10% reduction'.

during the 'TCM-90' experiment¹. However, we find that the data alone do not lead to superior dynamical forecasts. These are only obtained after careful preparation of initial conditions at $t=0$ hours, using a generalized inverse method developed originally for regional ocean modelling². The method finds the flow field that is the weighted least-squares best-fit to the dynamical equations, boundary conditions and other data over the preceding 24 hours, ($-24 \leq t \leq 0$), and also to the previous initial conditions valid at $t=-24$ hours. The huge number $0(10^5)$ of computational degrees of freedom in the fit is collapsed to a modest number $0(10^1)$, using the representer functions³ associated with the problem. There is one representer for each effectively independent datum. The weights in the fit must be chosen to be the reciprocals of prior estimates of error covariances. The representers then yield the posterior error covariances for the fit. Serial Fortran-77 code for the algorithm is easily adapted

to parallel computers⁴.

Our initial data at time $t=0$, prepared by the inverse calculation, were complemented with boundary data for $0 \leq t \leq 48$ hours obtained from a coarse-resolution global forecast. The results of 10 cases from the TCM-90 data set are shown in the figure. The mean forecast error for the inverse initialization method is compared with the Australian Bureau of Meteorology's standard initialization method⁵, and also with a statistical 'climatology persistence' method (CLIPER). The inverse method yields 14% improvements over the standard method at 24 hours, reducing to 10% at 48 hours. These improvements match those of the previous 30 years.

Finally we emphasize the simplicity of our dynamical model, which computes steering flows at only one pressure level, 500 hPa. Initialized by the generalized inverse method, this model also significantly outperforms more sophisticated, multilevel models.

A. Bennett

C. Hagelberg

College of Oceanic and Atmospheric Sciences,

Oregon State University,

Corvallis,

Oregon 97331-5503, USA

L. Leslie

Bureau of Meteorology Research Centre, Melbourne, Australia 3001

1. Elsberry, R. L. *Bull. Am. met. Soc.* **71**, 1305–1316 (1990).
2. Bennett, A. F. & Thorburn, M. A. *J. phys. Oceanogr.* **22**, 213–230 (1992).
3. Yosida, K. *Functional Analysis* 6th edn (Springer, New York, 1980).
4. Bennett, A. F. & Baugh, J. R. *J. atmos. ocean. Technol.* **9**, 426–433 (1992).
5. Holland, G. J. *et al. Weath. Forec.* **6**, 415–424 (1991).

Superantigen data

SIR — In their Letter "Evidence for a viral superantigen in humans" (*Nature*, **358**, 507; 1992), Monique Lafon and colleagues describe the rabies N-protein as a superantigen, stimulating V β 8-bearing human T cells and binding directly to major histocompatibility complex (MHC) class II antigens. However, some of this data may have been incorrectly interpreted. Western blotting (their Fig. 2c) clearly indicates that N-protein bound strongly to immunoglobulin heavy chain (M_r 50,000) run out on an SDS-PAGE gel following immunoprecipitation. Sadly, this binding was dismissed by that over-used term "non-specific", even though it was at least 10–20-fold stronger than the indiscernible MHC class II α -chain binding.

Further evidence for direct binding of N-protein to MHC class II-expressing B

cells was given in Fig. 2a and b of Lafon *et al.* However, the recipient line probably also expresses surface immunoglobulin. This possibility was not tested, though the negative control was a class II-, immunoglobulin-negative T-cell line not an MHC class II-, immunoglobulin-positive B cell. Could the receptor for rabies superantigenic N-protein in fact be IgH, as suggested by Western blotting? This would be a most striking finding and may indicate that certain superantigens do not need MHC class II molecules to interact with T-cell receptor V β domains but may use surface immunoglobulin on B cells instead.

John D. Fraser

Department of Molecular Medicine, University of Auckland,

Private Bag,

Auckland, New Zealand

LAFON REPLIES — Although pertinent, Fraser's point is only partially correct. Our data show that N-protein not only binds MHC class II molecules but also other cell surface molecules present on human B cells immortalized by Epstein-Barr virus (EBV). We draw this conclusion from the following experiments: (1) N-protein binds to the surface of murine fibroblasts transfected with MHC class II molecules but not to non-transfected ones, confirming that N-protein does bind to MHC class II molecules; and (2) N-protein not only recognizes EBV-transformed human B-cell lines expressing MHC class II molecules but also some negative MHC class II mutant cell lines, suggesting that N-protein reacts with additional surface molecules. These cell surface molecules could be the heavy chains of immunoglobulins.

It cannot be excluded that other receptors are expressed on the surface of B cells. It is noteworthy that the so-called "unspecific binding of superantigen to IgG" is visible in immunoblots presented in articles studying the reactivity of exotoxin superantigens with MHC class II (for example, J. A. Mollick *et al. Science* **244**, 817–820; 1989), indicating that binding to immunoglobulin G would be a general feature of superantigens rather than an exception.

Monique Lafon

Unité de la Rage

Institut Pasteur

25 rue du Dr Roux,

75724 Paris Cedex 15,

France

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

Echoes of D4 receptor repeats

SIR — Van Tol *et al.* have reported polymorphic variation of the dopamine D4 receptor in the human population¹. This variation is due to the presence in the gene of different numbers of a 48-base-pair repeat, which results in the expression of receptor molecules containing between two and seven copies of a 16-amino-acid motif in the third intracellular loop. To date, such an

repeats (696–743), and which appears to be a minor repeat.

These sequence relationships strongly suggest that the origin of the repeats in the human sequence predates the divergence of mammalian species as different as rat and man. In view of the presence in the rat of non-repeat sequences homologous to the human repeats, it seems most unlikely that the repeats are a more recent insertion into the human genome. The repeats therefore seem to have been conserved in the human population for a very long time, albeit with different numbers of copies. By contrast, the

they have a functional role, particularly if one considers that we have not found an individual with less than two repeats. In a related study (as yet unpublished) of several species of non-human primates, we confirm the presence of a repeat sequence with similarities at around 90% to the human repeat sequence.

Makoff's interpretation is not the only plausible explanation for the origin of the repeat sequence. (1) It could be argued that the repeat sequence found in humans became inserted (possibly through homologous recombination) after rat and human divergence. In this respect it is important that in an optimal alignment of the entire rat sequence with human D4 only the 36 base pairs immediately upstream to the human repeat sequence show good similarity with the rat sequence (100% at the amino-acid level), in contrast to the successive sequences (approximately 25%). (2) A primordial core sequence, made by a tandem duplication of what we call the flanking region, could have occurred in an ancestral mammal. On divergence of mammalian lineages the rat lineage may have lost this primordial sequence, while it was maintained in the human lineage and eventually elaborated to its current form.

Finally, it remains surprising that the rat has only a mere 63–71% similarity with the human D4 repeat consensus sequence, as given by Makoff — especially if one considers that outside this region there is much higher similarity (approximately 90%) between the two species. As a comparison, the human and rat D2 receptors display approximately 90% nucleic acid sequence similarity over their entire coding regions⁵. For the identification of the genetic mechanism responsible for the generation of the repeat sequence and/or divergence of the rat sequence, data from other species are needed.

Jay B. Lichter

Kenneth J. Livak

Du Pont Merck Pharmaceutical Co.,

PO Box 80400,

Wilmington, Delaware 19880-0400, USA

Jeffery Rogers

Southwest Foundation for Biomedical

Research,

Department of Genetics,

PO Box 28147,

San Antonio, Texas 78228, USA

James L. Kennedy

Hubert H. M. Van Tol

Clarke Institute of Psychiatry &

University of Toronto,

250 College Street,

Toronto, Ontario M5T 1R8, Canada

1. Van Tol, H. H. M. *et al.* *Nature* **358**, 149–152 (1992).

2. O'Malley, K. L. *et al.* *New Biol.* **4**, 137–146 (1992).

3. Van Tol, H. H. M. *et al.* *Nature* **350**, 610–614 (1991).

4. Corpet, F. *Nucleic Acids Res.* **16**, 10881 (1988).

5. Grandy, D. K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **86**, 9762–9766 (1989).

Human D4 repeat consensus 744–791, 792–839, etc.	A G C C G C C C G C G C C C C G G C C C T G C G G C C C A C T G T G C C C	Homology to repeat consensus (%)
Human D4 696–743	C CgC GCG CCC CGC Cga CCC aGc GGC Cct gGC cGg Cct tCc ccc aCG CC	65
Rat D4 675–722	C CgC GCG CgC CGC Cga CCC aGc GGC CgG gGC cGg CgG GtG TgG Gac CC	63
Rat D4 708–755	g CCG GtG tCG GaC CcT aCt CAG GGT CCC cTc tTc TCa GAt TGT cCG CC	67
Rat D4 756–803	t CCC tCa CCC AGC CTC Cgg aCG aGC CCC aCc GtC TCC AgC aGa cCa ga	65
Rat D4 798–845	A CCa GaG tCa GaC CTC tCt CAG aGC CCC TGC aGC CCC Ggg TGT cTG Ct	71

Alignment of the consensus sequence for the repeats in the human dopamine D4 receptor gene and similar regions in the rat D4 gene and elsewhere in the human D4 gene. Nucleotides which are the same as in the consensus sequence are shown in upper case. The numbers refer to the co-ordinates given for the human and rat sequences^{2,3}. Nucleotides 708–722 and 798–803 are in the two overlap regions and are shown in bold. The alignment used the program Multalin⁴ (Cherwell Scientific), which searched the complete rat D4 sequence for homologies with the human repeats. The four regions with the greatest degree of similarity were found to be immediately adjacent to each other. Optimal alignment between the human and rat sequences showed that the rat region 675–722 is very similar to human region 696–743, immediately upstream of the repeats.

arrangement appears to be unique for proteins within the seven transmembrane domain family; its significance is not yet clear.

Van Tol *et al.* stated that the rat homologue of the human D4 receptor does not contain this repeat region. This is not strictly true. At the DNA level, using the published rat D4 sequence of O'Malley *et al.*², I have identified four adjacent and partly overlapping regions which are 63–71% similar to the 48-bp repeat sequence of the human gene (see figure). All four regions are in the part of the rat sequence encoding the third intracellular loop and in a position analogous to that of the repeats in the human sequence. At the amino-acid level, the similarity is 38–50%, suggestive of poor functional conservation.

Despite their high DNA similarity to the human repeats, these regions in the rat sequence are not repeats themselves. Their levels of similarity are too low, varying from 33 to 60% for each pair. This compares with 90–96% among the human repeats. In addition to its close relationship to the human repeats, the 5' 36-bp of the first of the rat sequences shares 95% DNA identity (100% amino-acid identity) with a region in the human sequence³ immediately upstream of the

repetitive nature of the sequences has been lost in the rat. This argues for an important role for this part of the third intracellular loop of the human dopamine D4 receptor.

A. J. Makoff

Department of Cell Biology,

Wellcome Foundation,

Beckenham, Kent BR3 3BS, UK

LICHTER *ET AL.* REPLY — Makoff compares the polymorphic DNA repeat sequences in the human dopamine D4 receptor gene (DRD4)^{1,3} with a similar region in the rat DRD4 gene² and argues for an evolutionary origin of the repeat sequence that predates the divergence of rats and humans. We believe that these conclusions cannot yet be substantiated, and now have additional data that bear on the issue.

We find a tremendous amount of variation in the nucleotide sequence of the repeat region, above and beyond the variation we described¹. Sequence analysis of DNA from 87 unrelated individuals shows that there are at least three times more haplotypes than the seven forms described previously³. Despite the variation among repeats in humans, we find that the first and last repeats are invariant, which suggests that

Beyond methodology

John Ziman

In Search of a Better World: Lectures and Essays from Thirty Years. By Karl Popper. Routledge: 1992. Pp. 245. £25.

ON 28 July this year, a friend and I were discussing the work of Sir Karl Popper. We wondered how old he was. I looked in his autobiography. That very day was his ninetieth birthday! But the essays in this volume are far from 'last words'. Some are recent, but others go back to the 1950s. Their only common feature is that they were mostly written originally in German, and were evidently published together recently in that language, from which they have been specially (and very expertly) translated. But they cover the wide waterfront of his interests, and therefore invite what could be called a 'retrospective' of his work.

Popper would surely like to be known principally for his determined advocacy of criticism as an intellectual method. Following Socrates, he insists that the most important thing we know is that we know very little, and that only uncertainly. I am not sure that I agree factually with his assertion that "all the great natural scientists were intellectually modest", but it is a laudable fiction. For that reason, it is surprising that he says in one place in this book that he has "solved a whole string of really fundamental philosophical problems" — a belief other philosophers might fairly take leave to doubt.

Popper has actually influenced the climate of contemporary thought in three ways. Most importantly, in works such as *The Open Society and Its Enemies*, which was published in 1950, he thoroughly debunked political historicism. In the present essays, he continues the attack on intellectuals such as Plato, Hegel and Marx, who set themselves up as prophets on the basis of pretentious theories of social action. Popper's deflation of such gurus is based on a deep moral appreciation of

the chaotic forces and unfathomable sources of historical change. I strongly recommend the chapter entitled "Emancipation through knowledge" (broadcast in German, in Bavaria, in 1961), where he reaffirms the goal of the Enlightenment as giving meaning to our lives through our own efforts, rather than trying to discover meanings that could never be there *a priori*. In the light of his own critical stance, this can only be an article of faith, but it sits well with his repeated rejection of intellectual pessimism about the imminent fate of the world. I admire his courage in risking comparison with Voltaire's Dr Pangloss by publicly celebrating contemporary progress in the conquest of poverty, cruelty and disease.

Most readers of *Nature* know of Popper mainly for his philosophy of science. In the public mind, this is strongly associated with the concept of 'falsification' — that is, the power of awkward facts to put paid to cherished theories. This concept was developed by Popper in *Die Logik der Forschung*, published in 1934, although it did not become widely appreciated in the scientific world until it appeared in English as *The Logic of Scientific Discovery* in 1959. This eloquent work, and others that followed in the same vein, made sense of the intuitive strategy of good research by seeing it as a continual process of conjecture and refutation. As every scientist knows, a plausible web of hypotheses cannot be accepted as a serious account of scientific truth unless it can be fitted reasonably well to all the known facts, as well as to any new facts that research could be designed to reveal.

Interestingly enough, none of these essays mentions 'falsification' as such: presumably this concept is subsumed under more general notions of the need to subject scientific theories to critical tests. These notions, with their implication that all scientific knowledge is provisional and corrigible, are so natural in themselves, and so much part of our contemporary attitude to science, that it is difficult now to say how much they are due directly to Popper's advocacy. Certainly, many of us who read him in the 1960s were delighted that a philosopher had at last understood in principle how we often worked as scientists, and have been happy to cite his authority as a justification of our peculiar ways.

What these essays do show, however,

is Popper's disappointing failure to pursue this insight further. For example, his repeated advocacy of the search for 'truth' merely echoes the average working scientist's conviction that there is indeed just such a something to be pursued and captured. His only part in the serious philosophical debates that still go on about the status of scientific knowledge is to cheer on the 'realists' with strongly partisan slogans. In fact, many of these essays contain robotic sneers at all 'relativists', as if all the diverse, sincere, often wrong-headed but sometimes telling arguments that come under this heading were downright vicious and treasonable. As the champion of rational pluralism, Popper does himself a disservice by voicing such a sweeping condemnation of a whole variety of legitimate academic concerns. Incidentally, this is why everybody has ignored his attempt, in 1962, to discuss "The Logic of the Social Sciences". As the reprint here shows, it was embarrassingly insensitive to the impossibility of giving an account of social actions that is independent of the language and perceptions of the actors.

Finally, I would particularly commend his stress on the concept of "world 3" — that is, the world of books, works of art, manufactured goods and other "products of the human mind". This concept is not discussed much by philosophers or social scientists, and yet it could turn out to be the missing link between them. In effect, it provides a bridge between the subjectivity of the individual and the apparent objectivity of social institutions. Here again, Popper's instinctive recognition of an important idea is sound, but he holds back from the hard work of defining and refining it so that it can be used analytically as well as rhetorically.

Each of these essays speaks out in Popper's firm, clear, unambiguous voice. They do not pretend to cohere, but taken separately they are very readable and persuasive on important issues. Some readers may be interested in the chapter that provides the title to the book, where Popper presents his views on biological evolution — views that are so idiosyncratic that I do not feel qualified to comment on them. Otherwise, this book can scarcely be regarded as an important addition to his already impressive contributions to human understanding. □

John Ziman is at 27 Little London Green, Oakley, Aylesbury, Buckinghamshire HP18 9QL, UK.

■ Popper's life and works were celebrated by Hermann Bondi in a Commentary article published earlier this year to mark the philosopher's ninetieth birthday (*Nature* **358**, 363; 1992).

New in paperback

■ *Stephen Hawking: A Life in Science* by Michael White and John Gribbin. Penguin, £6.99. For a review see *Nature* **356**, 25 (1992).

■ *The Third Chimpanzee: The Evolution and Future of the Human Animal* by Jared Diamond. Winner of the Rhone-Poulenc Science Book Prize. Harper Perennial, \$12. For a review see *Nature* **352**, 676 (1991).

■ *German National Socialism and the Quest for Nuclear Power* by Mark Walker. Cambridge University Press, £13.95. See *Nature* **343**, 421 (1990).

Time signatures

Douglas Palmer

Terrestrial Ecosystems Through Time: Evolutionary Palaeoecology of Terrestrial Plants and Animals. Edited by A. K. Behrensmeyer, J. D. Damuth, W. A. DiMichele, R. Potts, H.-D. Sues and S. L. Wing. *University of Chicago Press: 1992. Pp. 568. £59.95, \$75 (hbk); £23.95, \$29.95 (pbk).*

THIS ambitious book represents the first attempt in print to discuss comprehensively the evolution of terrestrial ecosystems from Ordovician times, some 500 million years ago, through to the post-Pleistocene and the effects of humans. But it is much more than that: the first four chapters (roughly two hundred pages) are devoted to the methods of evolutionary palaeoecology, analysis of palaeoenvironments in general, taphonomic modes and a discussion of how extinct organisms can be characterized ecologically. In other words, the authors have gone to a great deal of trouble to define their terms and establish the ground rules for this new area. Justifiably so, because the project cuts across many interdisciplinary boundaries and involves a huge diversity of data and new information.

The book grew out of the first Evolution of Terrestrial Ecosystems Conference held near Washington DC in 1987. It is a carefully reworked synthesis of palaeoecological data and ideas set within the framework of geological time, presented by more than 30 invited contributors. And herein lies a fundamental methodological problem for palaeoecology: namely, the resolution of geological time. At best, Palaeozoic time cannot yet be resolved finer than 1 million years and even when we are dealing with the relatively recent past, for example the Pliocene (1.64–5.2 million years ago), the temporal resolution and correlation of strata between localities cannot generally be refined to within anything like the generational lifespans of ecology.

As Richard Potts and his coauthors of Chapter 7 on Late Cenozoic terrestrial ecosystems write: "much of the significant work in paleoecology depends upon temporal correlations, from which processes or causes of ecological change may be inferred". Even in the 'most recent' geological past such as the late Pleistocene (within the spectrum of carbon-14 dating), there are interesting and important questions about the role of humans in the megafaunal extinctions, when roughly 200 genera of large mammals disappeared within a few thousand years. To what extent can palaeoecology help to provide answers?

If the problems of temporal resolution and correlation can be overcome, then the study of evolutionary palaeoecology will benefit enormously. It will become an important tool that will help us to understand even better the immediate impact and long-term effects of human activity on Earth's ecosystems. Today, more than ten per cent of the total land on Earth is farmed, producing a dramatic change in the structures and dynamics of the global ecosystem, on a scale that is to be found only in the distant past. Suddenly, the major ecosystem changes of the Palaeozoic and Mesozoic take on a new relevance.

The authors do not try to duck the problems of temporal scaling, but use them to encourage future work with "carefully reconstructed local sequences of biotic, climatic, and geologic history" that are essential to understand "the behaviour of ecological systems over macroevolutionary time". This novel approach will hopefully provide a new impetus to the study of the geological history of Earth and its biota. This stimulating book deserves to be read well beyond the confines of the palaeontological community, as the themes that it addresses are important for us all. But to what extent we can learn to modify our future behaviour as a result of understanding the past remains to be seen. □

Douglas Palmer is at 31 Mawson Road, Cambridge CB1 2OZ, UK.

Selective attention

Steven Rose

Bright Air, Brilliant Fire: On the Matter of the Mind. By Gerald Edelman. *Basic Books/Allen Lane: 1992. Pp.280. \$25. £20.*

GERALD Edelman works on a broad canvas. His three earlier books, *Neural Darwinism*, *Topobiology* and *The Remembered Present*, published in rapid succession, offered nothing less than comprehensive theories of memory, development and the evolution of mind and of consciousness. His new book is intended as a somewhat more accessibly written résumé of the earlier three and as a challenge to many previous philosophies of mind. Edelman's theory has attracted both loyal support and strong opposition: the neurologist Oliver Sacks finds it inspirational; the molecular biologist Francis Crick, not averse to his own speculative flings on similar themes, has somewhat dismissively dubbed it

"Neural Edelmanism". Reviewers of the three earlier books in *Nature* were similarly divided (see *Nature* 331, 571, 1988; 336, 272, 1988; 343, 603, 1990).

As is now well known, Edelman's approach, which he categorizes as evolutionary and bases on darwinian principles, depends on what he abbreviates to TNGS, a "theory of neuronal group selection". TNGS holds that the fine details of the connectivity of the brain depend on the competitive selection of neurons and synapses during development, this selection occurring at the level of neuronal groups rather than individual cells and being driven by the pruning force of experiential contingency on the initial efflorescence of neuronal processes occurring early in the development of the vertebrate central nervous system. In these modest terms, such a model is uncontroversial. Developmental pruning and stabilization of synapses have been demonstrated and speculated about by Edelman and others, such as J.-P. Changeux, since the mid-1970s. The molecular mechanism Edelman proposes for this stabilization involves a class of glycoprotein cell adhesion molecules (N-CAMs), the discovery of which he himself was closely involved with; although in his books he simplifies their role (there are many other classes of molecule — such as the integrins — involved in synaptic recognition and stabilization), there is no doubt of the relevance of these molecules. Several research groups have recently demonstrated the involvement of the N-CAMs in the formation of memory.

But a theory of consciousness, or even of memory, requires more than molecular mechanisms, and to them Edelman adds an emphasis on what he calls the "reentrant" behaviour of neural circuitry — by which I assume he means, for it is nowhere defined in any of the books, the multiple feedback and feedforward loops through which groups of neurons are linked in space and time with their capacity for dynamic, plastic reorganization. Armed with this model, Edelman takes several fashionable theories of brain function, including, above all, the enthusiasm for "computational neuroscience" among neural-network modellers. The brain is not a computer, he reminds us often, forcibly and justifiably — a welcome emphasis, though he weakens the argument by then presenting his own learning robot with an inbuilt computer, called, in deference to his theoretical predilections, Darwin III. It is difficult for the uninitiated to appreciate from the account given in this book just how Darwin III's approach to learning differs from that of the modellers of whom Edelman disapproves.

TNGS contrasts Edelman's "selectionist" metaphor of neural development

with its "instructional" alternative. These terms have, of course, been taken over entirely from immunology, where the triumph of selectionism was to help Edelman towards his Nobel prize. I am not sure that the imported metaphor does service to the brain any more than the molecular details of the N-CAMs help the general thesis. Yet Edelman goes a stage further, punning immunological (and neuronal) selection with darwinian natural selection. Time and again he exhorts us to "population thinking" about the brain, and equates the evolution of natural populations of organisms with processes of neural development. But although the terms evolution and development share a common etymological origin, ontogeny does not simply recapitulate phylogeny, and it is hard to see how the elision helps our understanding of brain mechanisms. Populations of neurons are not equivalent to populations of organisms. Neurons develop, migrate, sprout synapses and die but, unlike any other cell in the body, they do not reproduce. And they do not compete through the differential survival of better-fitted offspring in any conceivable darwinian sense. Indeed, as sociobiologists are so keen to insist, darwinian selection is based on the individual not the population, and group-selection thinking is thus anathema to it. What value then, other than poetic, can Edelman's insistence on his double metaphor of selection have in understanding brain processes?

As the central figure in the Rockefeller (and now Scripps)-based Neurosciences Institute, Edelman has presided for many years over some of the most richly rewarding discussions of theory and experiment in the neurosciences anywhere in the world. This experience is reflected in the sweep and ambition of his writing, his insistence on an evolutionary neuroscience that ranges from molecular to cognitive processes and his sceptical dismissal of both crudely reductionist and nonmaterialist philosophies. Such strengths should not be lightly dismissed. But substituting selectionist for instructional models of neural development and rejecting an information-processing model of how brains work, useful steps though they are, still fail to do justice to the brain as an active, dynamic, self-organizing system. Karl Popper has spoken of replacing what he sees as the "passive Darwinism" of sociobiology with an "active Darwinism" in which the actor, the organism, plays a part in the development of its own future. Edelman's selectionist metaphor in its passivity denies this possibility.

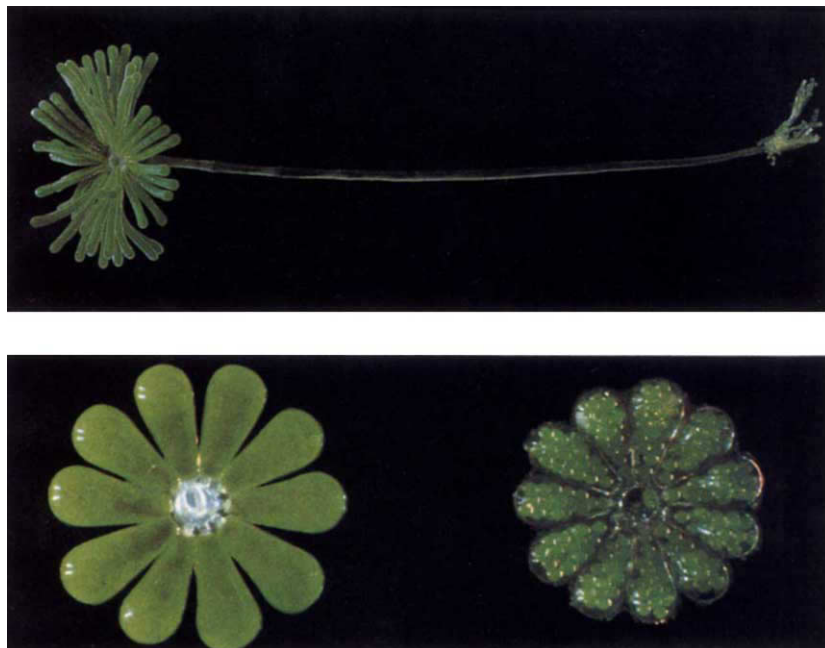
Edelman's earlier books, even for those who regarded them most highly, were seen as difficult and often opaquely written, as previous *Nature* reviewers

have pointed out. *Bright Air, Brilliant Fire* is an endeavour to overcome this problem, and from its title derived from Empedocles onwards the author has clearly worked hard to do so. Yet in many places the text can be comprehensible only to those who have read the earlier books (as with the discussion of Darwin III, for instance). The book isn't helped, in my view, by the early chapters with their potted references (complete with photographs) to Descartes, Darwin

and others, or by whatever editor suggested that the text would be lightened if, every few pages, the author inserted a bar-room joke or dubiously relevant anecdote. The faintly risible obscurity of the title's metaphor merely emphasizes the author's continuing problems in communicating accessibly. □

Steven Rose is in the Brain and Behaviour Research Group, Open University, Walton Hall, Milton Keynes MK7 6AA, UK.

Capping it all with algae



DASYCLADALES are a remarkable order of unicellular green algae widely found in the shallow waters of tropical or subtropical shores. Cells of the genus *Acetabularia* (top, $\times 2.2$) and the related genus *Polypysa* (bottom, $\times 20$) can grow to lengths of up to 20 cm and develop perfect radial caps containing globular packets of gametes. These cells were heralded as ideal objects for cell biological research in the heyday of J. Hämmerling *et al.*, but few of today's students even know of their existence. Hopefully this will change with the publication of the astonishing *Dasycladales: An Illustrated Monograph of a Fascinating Algal Order*, from which these pictures are taken. The authors, Sigrid Berger and Matthias J. Kaever, have given such lavish and loving attention to the production of this book that the word 'perfect' keeps coming to mind. The comprehensive text on the structure, ecology, evolution, taxonomy and palaeontology of these organisms will be invaluable to the dedicated phycologist; but what makes the book unique is its rich collection of photographs. Sadly this

does not include the striking immunofluorescence images of the actin and microtubule cytoskeletons of these cells (see, for example, D. Menzel and C. Elsner-Menzel, *Protoplasma* 157, 52; 1990). Nonetheless, the colour plates are technically and visually superb. They illustrate the extent to which the dasyclads have been indulging in an orgy of morphogenetic experimentation since at least the Cambrian era some 570 million years ago; the reader comes away with a whole new appreciation of what can be done with cell polarity, cell shape and genetic programming. This is a book that every biologist should purchase for a cherished nonscientific friend so that they can together share the fusion of nature and art that these creatures have perfected. Published by Georg Thieme, Stuttgart / Oxford University Press, New York. Price DM 298, \$175.

Ursula Goodenough

Ursula Goodenough is in the Department of Biology, Washington University, St Louis, Missouri 63130, USA

Wonders of the sky

Mark Saunders

The Solar–Terrestrial Environment. By J. K. Hargreaves. Cambridge University Press: 1992. Pp. 420. £50. \$79.95.

The Aurora: Sun–Earth Interactions. By N. Bone. Ellis Horwood: 1991. Pp. 156. £38, \$65 (hbk); £15.95, \$27.50 (pbk).

INTEREST in our solar–terrestrial environment (geospace) predates recorded history, but the main advances in knowledge have come since the dawn of the space age 35 years ago. The physics of this region, which consists of the Earth's upper atmosphere and magnetosphere, depends critically on the Sun and the solar wind. Ultraviolet radiation from the Sun photo-ionizes gases in the Earth's higher atmosphere, creating a zone of ionization called the ionosphere at altitudes ranging from 60 km to more than 300 km. The solar wind, a tenuous ($5 \text{ particles cm}^{-3}$), fast-flowing (400 km s^{-1}), highly electrically conducting plasma blowing continuously out from the Sun interacts with the Earth's magnetic field in subtle and complex ways. These interactions generate more than 5×10^{11} watts of power that drives the many physical processes, including aurorae, in the magnetosphere. Despite the considerable progress made over the past 35 years, few readable advanced-level texts have appeared. This deficiency is now partially remedied with the publication of these two books.

The textbook by J. K. Hargreaves is attractively produced, well illustrated, readable and interesting. It is intended for final-year undergraduates and first-year postgraduates taking courses in upper-atmospheric, ionospheric or magnetospheric physics. Mathematics is kept to an elementary level, the intention being to describe the basic concepts. To

this end, the author has done a good job, and the book is a more thorough and improved version of his previous volume *The Upper Atmosphere and Solar–Terrestrial Relations*, first published in 1979. The new book is sensibly divided into ten chapters, the first three introducing the basic physical principles and techniques for observing geospace. The main part of the book covers the neutral and ionized upper atmosphere and the magnetosphere and their associated structures, dynamics and disturbances. The text concludes with a brief chapter on technological applications.

The book does, however, suffer slightly from reflecting the special interests of the author instead of providing a coherent coverage of the subject as a whole. In particular, only one chapter is devoted to the solar wind and the magnetosphere, whereas the ionosphere receives three full chapters. And the material often seems a little outdated, failing to convey the true scope and excitement of present-day research. There are few recent references and no discussion of the many planned future developments.

Of the many fascinating phenomena in solar–terrestrial physics, aurorae are the most special for they can be observed without scientific instruments and have evoked awe and admiration for as long as Earth has been inhabited. The name 'aurora borealis' was suggested by Gassendi in 1621 and means 'northern dawn'. Its counterpart in the Southern Hemisphere (the aurora australis, or southern dawn) was predicted by de Mairan in 1731 and first recorded by Captain Cook in 1773. Our understanding of the aurora has developed slowly, and even today there remain important questions that are the topic of much research. *The Aurora* is an attempt to fill a notable gap in the literature. Most existing texts on the aurora are written for popular consumption or are suitable only for experienced research workers. This book bridges these extremes by providing a text accessible to amateur and professional astronomers, but also containing introductory material useful to research workers in solar–terrestrial physics. It reviews the new observations of the aurora made from satellites and from a

variety of ground-based stations.

The book is readable, adequately illustrated and well referenced. The attractive colour 'mini-atlas' of auroral types and forms is particularly welcome. There are detailed descriptions of the different types of aurorae (polar, mid-latitude,



Mary Evans

Auroral evidence — the aurora borealis as depicted in *Physique Populaire* (c. 1850).

radio aurora) and information on related high-atmosphere phenomena such as noctilucent clouds and airglow. Useful practical advice on auroral observations is also included.

Unfortunately, only a cursory description of the mechanism(s) behind the aurora is given. The bright, highly structured auroral forms such as arcs and rays are produced by the acceleration of electrons to energies of 1–10 keV at altitudes around 10,000 km. How this acceleration occurs is one of the most controversial topics in space physics and surely deserves mention. Most believe that decreases in electric potential parallel to the Earth's magnetic field lines are the cause, but new satellite observations (including those from the successful Swedish auroral probe Viking, which is surprisingly not mentioned in the text) are now causing many to question this opinion. □

Mark Saunders is in the Blackett Laboratory, Imperial College of Science, Technology and Medicine, London SW7 2BZ, UK.

Physics textbooks

■ *Newton to Einstein: The Trail of Light* by Ralph Baierlein. "An excursion to the wave-particle duality and the Special Theory of Relativity" for undergraduates, with emphasis on pivotal experiments. Biographical and historical sketches, and many exercises, complement the text. Cambridge University Press, £24.95, \$34.95.

■ *Introducing Einstein's Relativity* by Ray D'Inverno. Aims to provide students with a mathematical introduction, complete with many exercises. Clarendon/Oxford University Press, £22 (pbk).

Deflection and fragmentation of near-Earth asteroids

Thomas J. Ahrens & Alan W. Harris

The collision with Earth of near-Earth asteroids or comet nuclei poses a potential threat to mankind. Objects about 100 m in diameter could be diverted from an Earth-crossing trajectory by the impact of a rocket-launched mass, but for larger bodies nuclear explosions seem to be the only practical means of deflection. Fragmentation of the body by nuclear charges is less efficient or secure.

SEVERAL hundred asteroids and comet nuclei with diameters >100 m in Earth-crossing orbits have been discovered. Extrapolating this known population of near-Earth objects (NEOs) to those not yet discovered suggests that in total $\sim 2 \times 10^3$ objects ≥ 1 km in diameter lie on such orbits¹. The largest Earth-crossing asteroids have diameters of about 10 km. It is unlikely that any objects larger than 5 km remain undiscovered, but some as large as 3–5 km are probably still to be found.

Scientific interest in NEOs is great because it seems that many of these objects are main-belt asteroids which have been perturbed into terrestrial-planet-crossing orbits, thus constituting a large proportion of the impactors on terrestrial-planet surfaces². Objects as small as 5–10 m in diameter can be observed telescopically. Recently the object 1991 BA, 5–10 m in diameter, was seen to pass within $\sim 10^5$ km of the Earth³.

As the number distribution of different meteorite classes correlates poorly with asteroid type, as inferred from reflectance spectra of main-belt asteroids, it may be that the present terrestrial meteorite collection is a poor sample of the asteroid population. To gather more information, missions to near-Earth asteroids are currently being planned by NASA⁴.

New comets are brought into the swarm of NEOs by gravitational perturbation out of their orbits in the Kuiper belt and/or Oort cloud⁵. Some objects currently classed as near-Earth asteroids may in fact be devolatilized comets. Planet-crossing objects are removed from the population either through collision with a planet or by gravitational perturbations that eject them into hyperbolic orbits.

Earth-crossing asteroids have been recognized telescopically since 1932, when Reinmuth¹ discovered 1862 Apollo, and Gilbert's work⁶ on Meteor Crater, Arizona, and subsequent studies, made it apparent that the impacts of Earth-crossing asteroids and comets have produced the ~ 120 known meteorite impact craters on the Earth and virtually all the craters on the Moon. That the impact of any asteroid or comet with the Earth

could, in principle, have a catastrophic effect on life on the planet followed from the discovery of Alvarez *et al.*⁷ of the worldwide impact-ejecta dust layer at the Cretaceous/Tertiary (K/T) boundary, which is rich in platinum-group elements. It is now generally accepted that the impact of a bolide, ~ 10 km or more in diameter, with the Earth at the K/T boundary (65 Myr ago) gave rise to a great extinction of more than 50% of the known genera and probably 90% of all species.

In 1991, at the behest of the US House of Representatives, NASA conducted a series of studies of the asteroid-impact threat to the Earth⁸ and ways in which to avoid it⁹. The recent Near-Earth-Object Detection workshop⁸ quantified the hazards of Earth impactors, based in part on the results of an earlier workshop¹⁰. To judge from the estimated population of NEOs and their size distribution, objects with diameters of about 10 m strike the Earth almost annually, and although visible and audible for distances of 10^2 to 10^3 km, these objects largely break up and expend their energy (typically equivalent to 10 kton of TNT) in the atmosphere. Objects of about 100 m diameter include that of the 1908 Tunguska bolide (~ 10 Mton energy), which caused great damage even though it did not strike the Earth's surface. Impactors of this size are encountered about once every 300 years. Although they may induce local areas of devastation of $\sim 5 \times 10^3$ km², these objects lead to an annual probability of death for each individual of only 3×10^{-8} yr⁻¹ (ref. 8). Impactors of ~ 1 –2 km in diameter, which strike every 0.5 Myr, are thought⁸ to be the smallest that can produce catastrophic global effects (25% human mortality). An individual's annual probability of death from such an event is of the order of 5×10^{-7} , which is comparable to the risk of death in a commercial aeroplane accident. When viewed in this way, a rational approach to the threat ought to involve the expenditure of not more than a fraction of the amount committed to air safety and control. We believe that this would be in the range of 10^7 to 10^8 dollars per annum worldwide. It was concluded

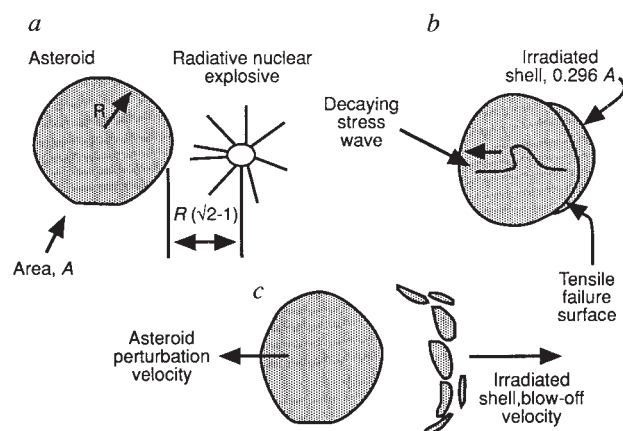


FIG. 1 How nuclear explosive radiation could be used to induce a velocity perturbation of $\sim 1 \text{ cm s}^{-1}$ in a near-Earth asteroid. *a*, Nuclear explosive designed to provide a substantial fraction, e , of its yield as energetic neutrons and γ -rays is detonated at an optimum height, $(\sqrt{2}-1)R$, above an asteroid. At this elevation the asteroid subtends 0.27 of the area of a unit sphere around the explosive, which irradiates 0.296 of the asteroid surface area. *b*, Irradiated to a depth of ~ 20 cm, surface material subsequently expands and spalls away from the asteroid, inducing a stress wave of several kilobars amplitude in the asteroid. *c*, Blow-off of the irradiated shell induces a velocity perturbation of $\sim 1 \text{ cm s}^{-1}$ in the asteroid.

at the Near-Earth-Object Detection workshop⁸ that funding at this level would vastly improve our knowledge of the population and distribution of near-earth objects by means of ground-based and possibly space-borne telescopes. The technologies that might be employed to divert asteroids can be expected to change so rapidly in the coming decades that it would be premature to conduct detailed engineering studies or to build prototypes at this stage.

Here we examine the energy requirements for perturbing the orbits of NEOs in order to deflect them from Earth impact. We consider NEOs in three size ranges 0.1, 1 and 10 km in diameter, whose annual fluxes on the Earth are respectively 10^{-3} , 10^{-5} and 10^{-8} (ref. 1). Objects significantly smaller than 0.1 km pose little threat because they do not penetrate the atmosphere intact. We then consider several strategies for both deflecting and explosively fragmenting such objects, using spacecraft-borne energetic devices that encounter the NEOs many years, or even decades before their projected Earth impact. To quantify our work, especially with regard to nuclear explosive cratering in the low-gravity asteroid environment, we employ recent studies of cratering under a range of gravitational strengths and atmospheric pressures¹¹ and impact ejecta scaling¹², which were not available to earlier studies^{13,14}. Short-warning responses, which might be necessary for new comets, have been discussed by Solem^{14,15}.

Deflection of near-Earth asteroids

Near-Earth asteroids may be deflected from Earth-crossing orbits by perturbation either perpendicular to or along the direction of motion—that is, by either speeding up or slowing down the orbital velocity relative to the Sun.

An increment of velocity Δv applied transversely to a circularly orbiting particle induces an eccentricity or inclination which results in an oscillation about the original orbiting point of amplitude

$$\delta \approx \frac{\Delta v}{v_0} a \quad (1)$$

where v_0 is the orbital velocity (30 km s^{-1} for the Earth) and a is the semimajor axis. To perturb a particle by $\delta \approx 1$ Earth radius, $R_{\oplus}$, the velocity increment required is

$$\Delta v \approx \frac{v_0 R_{\oplus}}{a} \approx 1 \text{ m s}^{-1} \quad (2)$$

To perturb a body on a time t short compared with the orbital period, a simple linear estimate suffices;

$$\delta = \Delta v t \quad (3)$$

To perturb a body by $1 R_{\oplus}$ in time t requires

$$\Delta v \approx \frac{R_{\oplus}}{t} \approx \frac{75 \text{ m s}^{-1}}{t \text{ days}} \quad (4)$$

The linear estimate reduces to the orbital oscillation after ~ 1 radian of orbital motion.

In contrast, an increment of velocity Δv applied parallel to the orbital motion changes the semimajor axis and, more importantly, changes the period, resulting in a long-term drift of the perturbed body from its original path. For an initially circular orbit, the mean drift velocity, $\Delta v'$ is in the opposite sense to and larger than Δv

$$\Delta v' = -3\Delta v \quad (5)$$

An even larger amplification occurs if the impulse is applied at the perihelion of an eccentric orbit. For an eccentricity of 0.5, $\Delta v' \approx -5\Delta v$. Thus, over a time long compared with the orbital period, an increment Δv applied parallel to v produces a deflection of

$$\delta \approx 3\Delta v t \quad (6)$$

Hence, for $1 R_{\oplus}$ deflection

$$\Delta v \approx \frac{R_{\oplus}}{3t} \approx \frac{0.07 \text{ m s}^{-1}}{t \text{ years}} \quad (7)$$

With a lead time of about a decade, a velocity increment as small as $\sim 0.01 \text{ m s}^{-1}$ could divert an asteroid from a collision course with the Earth.

Implementation of orbital diversion

We consider several means of inducing perturbations in the orbit of an Earth-crossing asteroid. A large mass fired to strike the asteroid would impart to it a change in kinetic energy, and thus in trajectory. Alternatively, a propulsion system attached to the object (a mass driver) could be used to alter its trajectory. Nuclear explosions could be used to cause deflection either through the influence of radiation from a nearby detonation or by the impulse imparted by ejecta from a cratering explosion on the asteroid surface.

Direct-impact deflection. Simple calculations suffice to show that it is feasible to deflect a small ($\sim 10^2 \text{ m}$ diameter) asteroid by direct impact of a spacecraft-borne mass. The kinetic energy delivered for even a modest encounter velocity ($\sim 12 \text{ km s}^{-1}$) of a two-stage-launched impactor spacecraft is transferred to the asteroid much more efficiently (70 to 80%)¹⁶ than the energy of surface explosions: the energy transferred at a velocity of 12 km s^{-1} is $70 \times 10^{10} \text{ erg}$ per g of impactor, which is much greater than typical chemical explosive energies (4×10^{10}

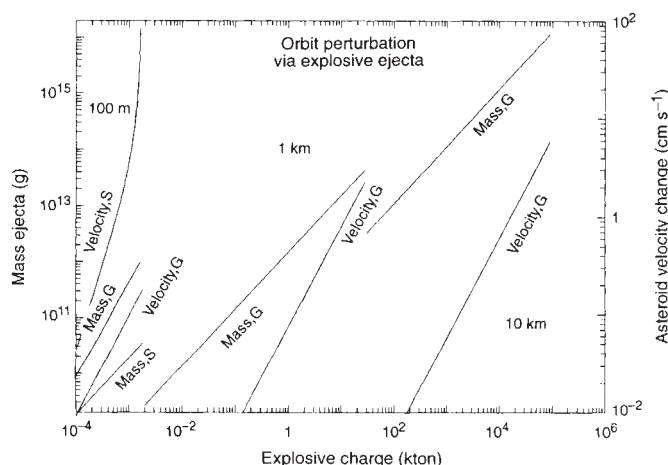


FIG. 2 Mass ejecta accelerated to more than escape velocity (left) for cratering explosive charges on surface of a 0.1-, 1- and 10-km diameter asteroid as a function of explosive yield. Plotted on the right is the resultant asteroid velocity change resulting from momentum conservation. G indicates gravity scaling and S strength scaling of crater size and dynamics. The curvature of the velocity plot for a 0.1 km diameter asteroid with strength scaling arises because a substantial fraction of the asteroid is ejected by an explosive crater in the 30 bar strength material.

erg g⁻¹). The ejecta throw-off from such an impact will suitably perturb the orbit. Although the cratering efficiency on a small (100 m diameter) asteroid (escape velocity 5 cm s⁻¹) is not known, extrapolation of small-scale studies (at high and low gravities) shows^{12,17} that it is likely to be ~10⁴ times the value for Earth of 2.8 tons of rock per ton of equivalent explosive yield¹⁸. For example, we calculate that the impact of a 200-kg projectile onto an asteroid 100 m in diameter, with a mass of 10⁶ ton and a density of 2 g cm⁻³, induces a gravity-limited crater with 10⁵ tons of ejecta having greater than escape velocity. This will perturb the velocity of the target asteroid by ~0.6 cm s⁻¹ even if one assumes (30-bar) strength, rather than asteroid gravity, is limiting crater formation¹⁹. Thus a cratering efficiency of at least ~10² tons of crater ejecta per equivalent ton of explosive is inferred.

For impactor deflection of small asteroids, it is possible that one could use an impactor device such as those developed for the US Strategic Defense Initiative, for example the Boeing Company's Lightweight Exoatmospheric Projectile. For larger asteroid diameters (~1 km), the increase in asteroid mass by factor of 10³ reduces the resulting perturbation velocity by the same factor. Moreover, the cratering efficiency declines owing to the increased gravity of the asteroid, and direct-impact deflection becomes impractical.

Mass drivers for deflection. As a long-term deflection strategy, one might imagine employing a mass driver system operating for several years. A lead time of three decades before Earth encounter would, from equation (7) require $\Delta v \approx 0.2$ cm s⁻¹. One possibility might be to deliver a reaction engine or 'mass driver' to an asteroid which would mine and eject material from part of the asteroid. Such a device operating on a 1-km-diameter asteroid over a decade timescale could provide the needed velocity perturbation. For an ejection velocity of ~0.3 km s⁻¹, the ejected mass necessary to produce a recoil of 0.2 cm s⁻¹ is

$$\Delta m \approx \frac{0.2 \text{ cm s}^{-1}}{0.3 \text{ km s}^{-1}} m_a = 7,000 \text{ ton}$$

where m_a is the asteroid mass. Although such a system might be technically feasible, it will become clear from what follows that nuclear energy offers a much more efficient solution.

Deflection by nuclear explosion radiation. By detonating above the asteroid (or comet) a nuclear explosive whose yield is largely in the form of neutrons, a large area of the surface can be irradiated²⁰. Subsequent blow-off of surface material in excess of the escape velocity can provide the necessary impulse for orbital deflection (Fig. 1). By detonating a charge at a normalized altitude $h/R = \sqrt{2} - 1 \approx 0.4$, where h is the altitude of the charge above the asteroid surface and R is the radius of the asteroid,

a maximum dose of $f_{\max} = 0.3$ times the total radiative yield is delivered to 0.3 times the unit area of a spherical asteroid. For a mean neutron cross-section of 10^{-24} cm², an assumed asteroid density of 2 g cm⁻³ and mean atomic weight of 25, we infer a characteristic neutron penetration depth of ~20 cm. Thus an asteroid volume corresponding to a 20-cm-deep surface shell covering 0.296 of the surface area is irradiated; for an object of radius 50 m and density 2 g cm⁻³, this will have a mass of 3.7×10^9 g. We further assume that the fraction, $e = 0.3$, of the explosive yield is delivered as neutron or other radiation and that this radiation is completely converted to internal energy, ΔE , per unit mass in the irradiated shell. The energy per kiloton of explosive yield delivered to the irradiated shell is thus

$$\Delta E = f_{\max} e (4 \times 10^{19}) \text{ erg} \quad (8)$$

where 4×10^{19} is the equivalent number of ergs in a metric kiloton of explosive yield. The irradiation will induce an average temperature rise of $<10^2$ K. This heating at constant volume of the shell will result in an increase in the pressure (per kiloton nuclear explosive yield) of

$$\Delta P = \gamma \rho \Delta E \quad (9)$$

where γ is the thermodynamic Gruneisen ratio, here assumed to be unity, and ρ is the asteroid or comet density, which we assume is 2 g cm⁻³. This irradiation occurs in less than the ~10² μs required for the sonic wave to travel through the shell. From equation (9), this energy density will raise the shell thermodynamic pressure to ~1.7 kbar (per kiloton nuclear explosive yield). As depicted in Fig. 1, this pressure increase accelerates the irradiated shell to the right, and a stress wave pulse is propagated to the left within the asteroid. The right-moving irradiated shell and left-propagating stress wave cause the irradiated shell to break away from the asteroid to conserve momentum (Fig. 1). The stress wave propagating to the left in the asteroid seems to be of low enough amplitude for little further destruction of the object to be likely. By assuming a compressional wave velocity, c_p , of 2 km s⁻¹ in the asteroid material, we find that the resulting outward particle velocity of the shell material

$$\Delta v_r = \Delta P / \rho c_p \quad (10)$$

is 44 m s⁻¹ kton⁻¹. Considering only the component of velocity along the direction between the explosive and the asteroid centre yields a reduced velocity of ~31 m s⁻¹ kton⁻¹. For the 50-m-radius asteroid, this velocity is much greater than the escape velocity of 5.3 cm s⁻¹. By conservation of momentum, the rebounding surface material translates into perturbation velocity of 11 cm s⁻¹ kton⁻¹ for the asteroid. For 1 and 10 km diameter objects, the comparable rebound velocities are 11×10^{-3} and

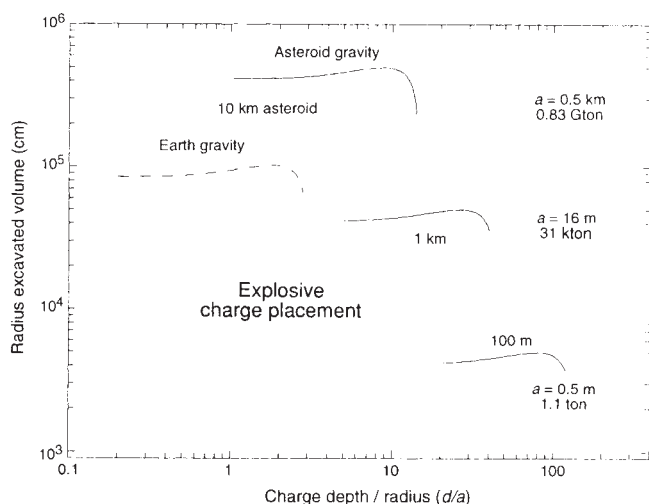


FIG. 3 Radius of excavated sphere of asteroidal material for 0.1-, 1- and 10-km asteroids, against normalized charge depth. The effect of nominal explosive yield is indicated for each size of asteroid. The effect of gravity is demonstrated by the curve labelled 'Earth gravity' which gives the excavated crater volume assuming terrestrial rather than asteroidal gravity for the 10 km asteroid case, where a 0.83 Gton explosive charge yields a crater radius of 5 and 1 km on the asteroid and Earth, respectively.

$11 \times 10^{-6} \text{ cm s}^{-1} \text{ kton}^{-1}$. If $e = 0.03$ rather than 0.3, however, these velocities will decrease by a factor of 10. Thus we conclude that deflection velocities of $\sim 1 \text{ cm s}^{-1}$ for asteroids of diameter 100 m, 1 km and 10 km, respectively, require detonation of 0.01–0.1 kton, 0.01–0.1 Mton and 0.01–0.1 Gton nuclear explosives.

Deflection by surface nuclear explosive. Another approach involving nuclear explosives is to use a surface charge to induce cratering on the asteroid. The thrown-off material effectively induces a velocity change in the asteroid and being highly dispersed, is not expected to be a hazard to the Earth. This method has the disadvantage that the asteroid may inadvertently be broken into large fragments which could be hazardous. For 0.1, 1 and 10 km diameter, we examine the nuclear explosive surface charge required to perturb asteroid velocity if gravity limits ejecta production, and if the asteroid is weak. For the case of a 0.1 km asteroid, it is conceivable that cratering processes are limited by asteroid yield strength. We examine this case also. The mass ejected per unit mass of explosive, when cratering is limited by gravity, is given by¹¹

$$\begin{aligned} \pi_v = & \{0.16 - 0.24(d/a_e)\pi_2^{0.194} + 2.11[(d/a_e)\pi_2^{0.194}]^2 \\ & - 2.38[(d/a_e)\pi_2^{0.194}]^3 \\ & + 0.663[(d/a_e)\pi_2^{0.194}]^3\} / [\pi_2^{0.581}] \end{aligned} \quad (11)$$

Equation (11) was obtained on the basis of small-scale laboratory centrifuge experiments under high gravity and reduced pressure, and large-scale nuclear explosive tests. It also describes a limited number of small-scale experiments conducted²¹ at reduced gravity and reduced atmospheric pressures. It is assumed that nuclear explosives can be assigned an equivalent TNT (high explosive) mass based on their yield. Here d and a_e are explosive burial depth and equivalent TNT explosive mass radius. Although equation (11) uses a radius (a_e) of an equivalent energy sphere of TNT, it is meant to describe cratering efficiency of buried nuclear explosives. Also, π_2 is defined as the gravity scaling parameter

$$\pi_2 = (m/\delta_e)^{1/3} g/Q \quad (12)$$

where m is the equivalent charge mass and δ_e is explosive charge density. For simplicity, we again assume that both charge density and asteroid density are 2 Mg m^{-3} . Q is the energy per unit mass of TNT ($4 \times 10^6 \text{ J kg}^{-1}$) and g is asteroid surface gravity. Because ejected material will alter the orbit of an asteroid only if it is thrown off at a velocity exceeding the escape velocity, we use the generalized equations of Housen *et al.*¹² to calculate the mass of ejecta, m_e , launched at speeds greater than escape velocity

$$m_e/(\rho R_c^3) = 0.32[2R/R_c]^{-0.61} \quad (13)$$

where R_c is the final crater radius. The mass of ejecta escaping the asteroid and the resulting asteroid velocity perturbation are shown as a function of surface explosive charge in Fig. 2. To relate R_c to m_e , we assume a conical crater with a depth to diameter ratio of 5. For surface explosions, this implies that ~ 0.1 , 10^2 and 10^4 kton of explosive energy are needed to perturb the orbital velocity of 0.1, 1 and 10 km diameter objects by $\sim 1 \text{ cm s}^{-1}$. Moreover, for a 100-m asteroid, strength scaling¹⁹ may apply. In the case of an effective yield strength of 30 bars, Fig. 2 indicates that only 500 kg would be required to perturb a small asteroid by 1 cm s^{-1} .

Thus, at best, surface explosions are no better than radiative stand-off explosions, and the requirements are more uncertain.

Fragmentation and dispersal

Small-scale fragmentation experiments on solid rocks²² demonstrate that the bulk of the fragments of a collisional disruption have velocities of $\sim 10 \text{ m s}^{-1}$. The 'core' or largest fragment, however, has a differential velocity of no more than $\sim 1 \text{ m s}^{-1}$ (ref. 22). From equation (4), if the body is fragmented ~ 75 days before Earth encounter then most of the $\geq 10 \text{ m}$ fragment will

still strike the Earth. For a small object (0.1 to 1 km), dispersal of the bulk of the fragments into the Earth's atmosphere may be sufficient, as long as no fragments $\geq 10 \text{ m}$ are allowed. A really large object ($> 1 \text{ km}$) would have to be fragmented one or more orbits before intersection with the Earth to ensure that most fragments missed the Earth. In general, the debris cloud would spread along the orbit according to equation (6) and in the transverse direction according to equation (1). For a characteristic ejecta velocity of 10 m s^{-1} , the debris cloud would be $\sim 10 R_\oplus$ in radius (with some oscillation about the orbit) and grow in length by $\sim 200 R_\oplus$ per orbit period. If the asteroid were destroyed one orbit before encounter, the Earth might encounter as little as 0.1% of the debris. But more conservatively, if many large fragments with $\Delta v \leq 1 \text{ m s}^{-1}$ remained, as much as 10% of that mass might be intercepted. Fragmentation is likely to be a safe choice only for long lead-times (decades) or small bodies, for which the fragments may still hit the Earth.

Catastrophic disruption is generally defined as fragmentation in which the largest fragment is $\leq 1/2$ the mass of the original body. The energy density to accomplish this decreases with increasing size of body and becomes uncertain when extrapolated to bodies 1–10 km in size²³. For the present purpose, however, we are interested in the energy density necessary to break up an asteroid so that all fragments are $\leq 10 \text{ m}$ in size. This is obviously a higher energy density than that needed just to 'break in two', and we suggest that it is of the order of the energy density needed to 'break in two' a 10 m object, $\sim 10^7 \text{ erg g}^{-1}$.

Because of the large energy requirements to fracture a well consolidated asteroid, only nuclear explosives are considered. To relate energy density to radius for a completely coupled (buried) nuclear charge, we use the empirical relations of Cooper¹⁶ for shock-induced particle velocity, v against energy-scaled radius ($r/kT^{1/3}$). For hard (mainly igneous) terrestrial rocks

$$\ln_{10} v(\text{m s}^{-1}) = 5.233 - 2 \ln_{10} (r/kT^{1/3}) \quad (14)$$

where the r radius is hydrodynamically scaled by the one-third power of explosive yield (expressed in kilotons TNT), $kT^{1/3}$. Similarly, for soft rocks

$$\ln_{10} v(\text{m s}^{-1}) = 4.590 - 2 \ln_{10} (r/kT^{1/3}) \quad (15)$$

As the shock-wave energy per unit mass is equal to v^2

$$E_{\text{frac}} = v^2(r, kT^{1/3}) \quad (16)$$

where v^2 can be specified from equation (14) or (15), and $E_{\text{frac}} = 10^3 \text{ J kg}^{-1} = 10^7 \text{ erg g}^{-1}$. On substituting equation (15) into equation (16) for 1 kton, we find $r = 35 \text{ m}$. Thus, a 1 kton explosive charge is expected to fragment a 35-m-radius sphere of rock, if the explosive is placed well within the asteroid. Also, a 1 Mton charge of explosive will fragment a 350-m-radius sphere of rock and 1 Gton of explosive will fragment 3.5 km of rock. In contrast, for hard rock, equation (14), which describes less attenuative rock, gives the radius of fracture as 74 m for a 1 kton explosion. From equation (15), to deliver 10^7 erg g^{-1} to 0.1, 1 and 10 km diameter asteroids requires 3 kton, 3 Mton and 3 Gton, centrally placed.

The above discussion is based on the premise that the charge is buried deep enough to produce optimum fragmentation. Some nuclear charge burial is desirable, as surface-exploded nuclear charges transfer only a small fraction of their energy (0.2 to 1.8%) to rock, by radiative and hydrodynamic coupling²⁴, whereas a much larger fraction of the energy of a deeply buried charge is transferred into the surrounding rock.

Figure 3 shows the charge depth for different d/a values and yield required completely to excavate asteroids of 100, 1,000 and 10,000 m diameter. The yields required for an excavating charge are reduced—by a factor of 3×10^3 for a 0.1 km asteroid, to a factor of 4 for a 10 km asteroid—relative to those calculated for fragmentation. These charges are in the 0.15–3 kton range

for a 100 m asteroid, 0.007–3 Mton for a 1-km asteroid, and 0.3 to 3 Gton for a 10-km asteroid. The effect of gravity on the radius of excavated volumes is seen to be substantial. Notably, the optimum depth of charge (producing largest radius of excavated volume) decreases with increasing asteroid size and surface gravity. Figure 3 also shows that the radii of excavated volumes decrease by a factor of up to 5 in going from the gravity of a 10 km diameter object (0.3 cm s^{-2}) to that of the Earth (982 cm s^{-2}). Dispersal seems to require about the same energy as deflection, and is also benefited by charge burial. Hence, asteroid deflection, rather than destruction by fragmentation, appears to be the most efficient strategy.

Conclusions

We have shown that to deflect Earth-crossing objects (asteroids and comets) from a collision course, velocity perturbations much smaller than 1 m s^{-1} are sufficient for lead times of a decade or more. For objects discovered only as they approach on a collision course, however, the necessary velocity perturbations are tens to hundreds of metres per second; for larger bodies the energy required for this is prohibitive and the perturbation impulse would disrupt the body.

We also note that perturbation of an object is more effectively accomplished by applying a change in velocity, Δv , along its original orbit, inducing a change in the orbital period and hence the position along the orbital path. Applying an impulse at perihelion (for an eccentricity of 0.5) produces a velocity perturbation that provides a larger deflection ($\sim 5\Delta v t$ at time t).

For a $\sim 100 \text{ m}$ diameter asteroid, the kinetic energy of 10^2 to 10^3 kg impactors, intercepting at 12 km s^{-1} will be sufficient to crater and launch ejecta which, in the low-gravity environment of these objects, will induce velocity perturbations of $\sim 1 \text{ cm s}^{-1}$. For larger asteroids, this method of deflection seems impractical because of the large mass of impactors required. Mass drivers must launch $\sim 10^3$ to 10^4 tons of material from a 1-km asteroid over 30 years to deflect it from Earth impact. Nuclear explosive irradiation may be used to blow off a 20-cm shell encompassing ~ 0.3 times the asteroid surface area if the charge is exploded at an optimum height of $h/R = \sqrt{2} - 1$. Minimum charges of

0.01, 10^2 and 10^4 kton of explosives are required to perturb by 1 cm s^{-1} the velocity of 0.1-, 1- and 10-km asteroids, respectively; less radiatively efficient explosives may require an order of magnitude more explosive yields. Surface charges of 10^{-2} , 10 and 10^5 kton may be used to eject crater material at velocities greater than local escape velocity, and hence perturb 0.1-, 1- and 10-km diameter asteroids by a velocity increment of $\sim 1 \text{ cm s}^{-1}$. Burial of nuclear charges to induce fragmentation and dispersal requires *in situ* drilling, which is difficult on a low-gravity object. Complete excavation (that is, production of a volume of ejecta equal to the volume of the original asteroid) of 0.1-, 1- and 10-km diameter asteroids (working only against local gravity) requires optimally buried charges with energies of ~ 1 ton, 30 kton and 0.8 Gton respectively.

In general, deflection methods seem to be preferable to fragmentation approaches because the former do not require charge burial. For a small (100 m) asteroid, deflection using a kinetic-energy impact is technically feasible and does not involve the politically complex issue of placing nuclear explosives on a spacecraft. For asteroids in the 1–10-km range, which includes the largest Earth-crossing objects, only nuclear-based options are practical, of which deflection by nuclear explosive radiation seems to be the simplest method. This method requires less detailed knowledge of the physical characteristics of the Earth-crossing object, and the development of the charges required to deflect large Earth-crossing objects seems to be feasible technologically.

Finally, we note that, although further study of the feasibility of diverting asteroids may be warranted, we do not believe it is appropriate to conduct engineering designs of systems at this stage because of the low probability of impact of hazardous asteroids, the high cost in the face of a low risk factor, and the rapid changes that are to be expected in defence systems technology. $\square$

Thomas J. Ahrens is at the Lindhurst Laboratory of Experimental Geophysics, Seismological Laboratory 252-21, California Institute of Technology, Pasadena, California 91125, USA. Alan W. Harris is at the Jet Propulsion Laboratory, California Institute of Technology, 4800 Oak Grove Drive, Pasadena, California 91109, USA.

- Shoemaker, E. M., Wolf, R. F. & Shoemaker, C. S. *Geol. Soc. Am. spec. Pap.* **247**, 155–170 (1990).
- Binzel, R. P., Xu, S., Bus, S. J. & Bowell, E. *Science* **257**, 779–782 (1992).
- Scotti, J. V., Rabinowicz, D. L. & Marsden, B. G. *Nature* **354**, 287–289 (1991).
- Veveka, J. & Harris, A. W. *Near Earth Asteroid Rendezvous (NEAR) Science Working Group Rep. JPL-86-7* (Jet Propulsion Laboratory, Pasadena, California, 1986).
- Weissman, P. R. in *Global Catastrophes in Earth History* (eds Sharpton, V. L. & Ward, P. D.) 171–180 (Geol. Soc. Am. spec. Paper, 1990).
- Melosh, H. J. *Impact Cratering*, 1–245 (Oxford Univ. Press, New York, 1989).
- Alvarez, L. E., Alvarez, W., Asaro, F. & Michel, H. V. *Science* **208**, 1095–1108 (1980).
- Morrison, D. *The Spaceguard Survey: Report of the NASA International Near-Earth-Object Detection Workshop* (January 25, 1992) (1992).
- Rather, J. D. G., Rahe, J. H. & Canavan, G. *Summary Rep. Near-Earth-Object Interception Workshop* (NASA Headquarters, 1992).
- Shoemaker, E. M. *NASA Workshop, Collision of Asteroids and Comets with the Earth: Physical and Human Consequences*, Snowmass, Colorado, July 13–16, 1981 (1983).
- Housen, K. R., Schmidt, R. M., Voss, M. E. & Watson, H. E. *DNA-TR-92-24* (Boeing Company, 1992).
- Housen, K. R., Schmidt, R. M. & Holsapple, K. A. *J. geophys. Res.* **88**, 2485–2499 (1983).
- MIT Students. *Project Icarus* 1–162 (MIT Press, Cambridge, Massachusetts, 1968).
- Solem, J. C. LA-UR-231 (Los Alamos National Laboratory, 1992).
- Solem, J. C. LA-UR-91-3765 (Los Alamos National Laboratory, 1991).
- Smither, C. L. & Ahrens, T. J. in *Proc. Int. Conf. Asteroids, Comets, and Meteors, June 24–28, 1991* (eds Harris, A. W. & Bowell, A.) (Lunar planet. Sci. Inst., Houston, 1992).
- Holsapple, K. A. *A. Rev. Earth planet. Sci.* **22** (in the press).
- Cooper, H. F. Jr in *Impact and Explosion Cratering* (eds Roddy, D. J., Pepin, R. O. & Merrill, R. B.) 11–44 (Pergamon, New York, 1977).
- Holsapple, K. A. & Schmidt, R. M. *J. geophys. Res.* **87**, 1849–1870 (1982).
- Hyde, R. A. *UCID-20062* (Lawrence Livermore National Laboratory, 1984).
- Johnson, S. W., Smith, J. A., Franklin, E. G., Moracki, L. K. & Teal, D. J. *J. geophys. Res.* **74**, 4838–4850 (1969).
- Nakamura, T. & Fujiwara, *Icarus* **92**, 132–146 (1991).
- Housen, K. R. & Holsapple, K. A. *Icarus* **84**, 220–253 (1990).
- Schmidt, R. M., Holsapple, K. A. & Housen, K. R. *DNA-TR-86-182* (Boeing, Seattle, 1986).

ACKNOWLEDGEMENTS. T.J.A. benefited from the technical discussions held at the NASA Workshop on Near Earth Object Interception, 14–16 January, 1992. We thank R. Schmidt, K. Holsapple and J. C. Solem for their preprints. This research was supported by NASA.

Molecular analysis of the association of HLA-B53 and resistance to severe malaria

Adrian V. S. Hill*, John Elvin*, Anthony C. Willis†, Michael Aidoo‡, Catherine E. M. Allsopp*, Frances M. Gotch*, X. Ming Gao*, Masafumi Takiguchi§, Brian M. Greenwood‡, Alain R. M. Townsend*, Andrew J. McMichael* & Hilton C. Whittle‡

* Molecular Immunology Group, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

† MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

‡ Medical Research Council Laboratories, Fajara, Banjul, The Gambia, West Africa

§ Institute of Medical Sciences, University of Tokyo, Minato-ku, Tokyo 108, Japan

The protective association between the human leukocyte antigen HLA-B53 and severe malaria was investigated by sequencing of peptides eluted from this molecule followed by screening of candidate epitopes from pre-erythrocytic-stage antigens of *Plasmodium falciparum* in biochemical and cellular assays. Among malaria-immune Africans, HLA-B53-restricted cytotoxic T lymphocytes recognized a conserved nonamer peptide from liver-stage-specific antigen-1 (LSA-1), but no HLA-B53-restricted epitopes were identified in other antigens. These findings indicate a possible molecular basis for this HLA-disease association and support the candidacy of liver-stage-specific antigen-1 as a malaria vaccine component.

FOR 25 years, HLA associations with diseases have provided clues to pathogenesis but the molecular basis of these associations has not been defined^{1,2}. The determinant selection hypothesis, that associations result from the ability of a particular HLA type to present a critical antigenic peptide, has been difficult to investigate because, for most disease associations, the relevant antigen is unknown. Recently, the identification of characteristic sequence features in peptides eluted from HLA class I molecules^{3,4} suggested that the relevant antigen might be identifiable by assessing cellular immune responses to peptides containing such motifs among antigens which are candidates for mediating HLA-disease associations. The finding of HLA associations with severe *P. falciparum* malaria⁵ provided an opportunity to test such a 'reverse immunogenetic' approach to identifying an immune response to the malarial parasite of protective importance.

Numerous antigens from *P. falciparum* have been cloned and sequenced in efforts to develop a malaria vaccine, but progress has been impeded by uncertainty as to which, if any, of these antigens elicits a protective immune response⁶. We analyse here the association between the HLA class I antigen, HLA-B53, and protection from severe malaria. We have hypothesized that this might be mediated by HLA class I-restricted cytotoxic T lymphocytes (CTL) acting during the liver-stage of the parasite's life cycle⁵. Immunization studies using irradiated sporozoites have indicated that responses to pre-erythrocytic-stage antigens, those expressed by sporozoites or liver-stage parasites, can provide complete immunity both in rodents⁷ and in humans⁸, and that similar protective mechanisms, of which CTL appear to be an important component⁹⁻¹⁹, may operate in each species. The impracticality of large-scale immunization with irradiated sporozoites has led to efforts to identify protective pre-erythrocytic antigens. But identification of cytotoxic T cells in humans naturally exposed to malaria has been problematic^{15,16}, partly because of the lack of a suitable *in vitro* restimulation system to generate CTL.

By eluting self-peptides from HLA-B53 we identified a sequence motif for bound peptides and have synthesized 60 octamer, nonamer or decamer peptides bearing this motif from the four pre-erythrocytic-stage *P. falciparum* antigens that have been cloned and sequenced²⁰⁻²⁵; each of these antigens has been

used in or is currently awaiting human vaccine trials. One or more peptides from each antigen bound to HLA-B53 in an HLA assembly (binding) assay²⁶ and these were tested further using lymphocytes from Africans naturally exposed to malaria. None of the peptides from the three sporozoite antigens, which have been the focus of most previous work¹¹⁻²³, elicited CTL responses. But a nonamer peptide from liver-stage-specific antigen-1 (LSA-1)^{24,25} elicited secondary CTL responses in HLA-B53-positive individuals and sequence analysis showed this to be a conserved epitope in the local parasite population. In contrast, a similar approach for the HLA-B35 molecule identified responses mainly to polymorphic epitopes, variants of which escape CTL recognition. This suggests a possible molecular basis for the association of HLA-B53 with resistance to severe malaria, and identify LSA-1 as an important antigen for attempts to induce CTL-mediated immunity to this infectious disease.

Peptide elutions

Self peptides were acid-eluted³ from HLA-B53 (HLA-B*5301), separated by high-performance liquid chromatography (HPLC) and sequenced. The pool sequence (Table 1) showed a predominant signal for proline at position 2 indicating that most peptides bound to HLA-B53 have this 'anchor' residue at position 2. A similar approach was taken for HLA-B35 (HLA-B*3501), which was not significantly associated with altered resistance to severe malaria in the Gambian case-control study of malaria susceptibility⁵. HLA-B35 also showed a strong preference for proline at position 2 of bound peptides (Table 1), probably because of its sequence identity to HLA-B53 in the region of the B pocket²⁷ of the molecule. But HLA-B35 showed a predominant tyrosine residue at position 9 of bound peptides: no anchor residue was identified at this position for HLA-B53.

These peptide motifs allowed us to search the sequences of known *P. falciparum* antigens for potential epitopes for HLA-B53 and -B35. We reasoned that a malaria antigen (or antigens) which could interact with HLA-B53 and account for the disease association should be expressed early in the liver-stage of the parasite's life cycle: human erythrocytes do not express class I molecules²⁸, and enhanced CTL lysis of macrophages that have engulfed blood-stage parasites is unlikely to benefit the host.

Furthermore, comparison of radiation doses required to induce protection in the irradiated sporozoite model of pre-erythrocytic immunity has indicated that antigens expressed early in development in the liver are of most importance for protection²⁹⁻³¹. So, peptides conforming to these motifs were identified in the three sequenced sporozoite antigens (circumsporozoite protein (CSP), TRAP and Pfs16) and the only sequenced liver-stage-specific antigen (LSA-1) from *P. falciparum*.

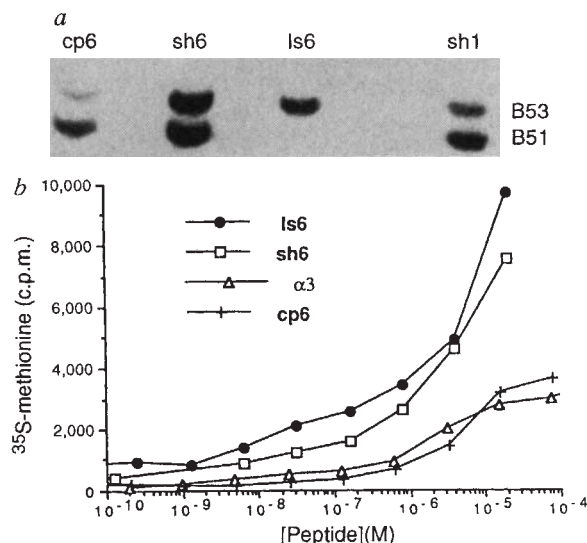


FIG. 1 HLA assembly assay. Stable assembly of HLA class I molecules in the mutant cell line T2 is dependent on binding by peptides⁵⁸⁻⁶⁰ and this phenomenon can be used as a peptide binding assay²⁶. We have found, using this assay, that all optimized CTL epitopes for other class I molecules (HLA-A2, D^b and K^b) have bound with high affinity, and the assay has recently been used to identify a HIV-gag CTL epitope in mice by screening of potential epitopes fitting the D^b motif⁶¹. a, Autoradiograph of a one-dimensional isoelectric focusing gel analysis showing binding or stabilization of HLA-B53 and -B51 by peptides cp6, sh1 and sh6, and of HLA-B53 by the ls6 peptide. Peptide binding is associated with stabilization of assembled class I molecules which appear as strong bands in the autoradiograph. In the absence of peptide (lane 2) class I molecules lose conformation, dissociate and are not precipitated by specific monoclonal antibodies. The peptides tested were, from left: cp6, saline negative control, sh6, ls7, ls6, sh5, cp22, sh1. Cp6 bound more strongly to HLA-B51 than to HLA-B53 but the allelic variants of this peptide with single amino-acid substitutions at position 4, cp6.1 and cp6.2, bound more strongly to HLA-B53 and very poorly to HLA-B51 (data not shown). b, Titration curve of the assembly of HLA-B53 with varying concentrations of the peptides ls6, cp6, sh6 and α3, YPAEITLYW, which was the major self-peptide eluted from HLA-B53. The y-axis represents the quantity of assembled class I heavy chain precipitated after addition of peptide, the x-axis the concentration of peptide added. The ls6 and sh6 peptides and the cp6 and α3 peptides were titrated in separate experiments. All four show 50% of maximum assembly of HLA-B53 in the micromolar concentration range (10⁻⁵–10⁻⁶ M), as does an HIV-2 nonamer epitope for HLA-B53 identified recently (F.M.G. *et al.* unpublished data). This is a higher concentration range than for HLA-A2-binding epitopes and some HLA-B51-binding peptides, but comparable to an epitope³⁵ for the mouse K^b molecule (ref. 61 and unpublished data).

METHODS. The assembly⁶¹ assay was done as described elsewhere^{26,58,60,61}. Briefly, the mutant cell line T2-B53 was derived from T2 cells of HLA type, HLA-A2, -B51, by transfection with a genomic clone of HLA-B53 using hygromycin selection. Peptides were synthesized by Cambridge Research Biochemicals, Cheshire, UK using their multiple peptide synthesis service or, subsequently, in Oxford using the same procedure and apparatus. Peptide concentrations were determined with a Micro BCA colorimetric assay (Pierce). Peptides were added to lysates of ³⁵S-methionine-labelled cells and incubated overnight at 4 °C⁵⁸. Stable assembled class I molecules were then precipitated with monoclonal antibodies W6/32 (ref. 56; reactive with all assembled class I molecules) or MHM5 (ref. 62; reactive with assembled HLA-B but not HLA-A molecules). The precipitated class I molecules were separated by one-dimensional isoelectric focusing, visualized by autoradiography and quantified as described^{26,61}. All peptides were initially tested at 4 μM concentration, and those found to bind titrated to estimate binding affinities.

HLA assembly assay

A total of 60 peptides with proline at position 2 were synthesized (Table 2), to determine whether these were HLA-B53-restricted CTL epitopes in malaria-immune Africans. But this large number of peptides would have prevented comparison of responses to all peptides in the same individual, unless very large blood volumes were donated. So, we first used an HLA assembly assay²⁶ to identify a subgroup of these peptides which bound with high affinity to HLA-B53. Only eight peptides from the four malaria antigens bound in this assay, all of which had hydrophobic or aromatic residues at position 9 (Table 2, Fig. 1). For HLA-B35 peptides, the additional requirement for a terminal tyrosine limited the number of potential epitopes to be tested to 10 (Table 2).

HLA-B53-restricted CTL

Most adults in areas of hyperendemic malaria have immunity to malaria which, although not completely effective in preventing parasitaemia and occasional symptomatic episodes, is strongly protective against life-threatening malaria³². We studied healthy adult Gambian villagers who had the HLA-B53 antigen. CTL were detected to none of the peptides from the three sporozoite antigens, but three out of six individuals tested responded to the ls6 peptide from LSA-1 (Fig. 2). This CTL lysis was HLA-B53-restricted, peptide-specific and inhibitable by anti-CD8

TABLE 1 Pool sequences of peptides eluted from HLA-B53 and HLA-B35

HLA-B53 pool sequence									
	1	2	3	4	5	6	7	8	9
Anchor	P								
Strong				E	I				
Weak	S		F	I	L	Y			
	Y		K	L					
	F		N	Q					
	M		Q						
HLA-B35 pool sequence									
	1	2	3	4	5	6	7	8	9
Anchor	P								
Strong			F						Y
Weak	F		E	E					
	Y		I	K					
	L		N						
	M		Q						
			Y						

The sequences of these HLA molecules differ from each other at only five amino acids, all in the α1 domain at the end of the cleft which binds the carboxy terminus of the peptide^{52,53}. Anchor, strong and weak signals are classified as described by Falk *et al.*³ (single-letter amino-acid code). Peptides from both molecules have proline as an anchor residue at position 2, but only HLA-B35 has a clear anchor, tyrosine, at position 9. Partial sequences of several individual peptide peaks eluted from HLA-B53 (not shown) and a single complete sequence from the largest individual peak, YPAEITLYW, all showed proline at position 2. The latter sequence was identified as part of the α3 domain sequence of HLA-B53, as well as some other class I molecules. In contrast, peptides eluted by the same method from an HLA-B8 transfectant showed anchor residues at positions 3 and 5 (ref. 54). **Methods.** To determine whether particular sequence features were found in peptides presented by HLA-B53 (HLA-B*5301), as has been described for other class I molecules³⁴, we used the cell line Hmy-B53 (ref. 55): this was derived by transfection of the cell line CIR, which lacks HLA-A and -B molecules, with a genomic clone of HLA-B53. 1.5 × 10¹⁰ Hmy-B53 or Hmy-B35 cells⁵⁵ were pelleted and lysed and HLA class I molecules purified with the monoclonal antibody W6/32 (ref. 56) on an immunoaffinity column as described³. After acid elutions in 0.1% TFA, the supernatants were dried by vacuum centrifugation and separated by reversed-phase HPLC using a Brownlee Aquapore RP300 column (C8, 100 × 2 mm) and Severn Analytical equipment. Peptide peaks³ were pooled and sequenced using an Applied Biosystems 473A protein sequencer and Applied Biosystems model 610 data analysis software.

antibodies. We then sequenced parasite DNA from nine Gambian isolates (Fig. 2, legend): in each, the Is6 epitope was identical to the published sequence²⁵, indicating that it is largely or completely conserved in this population.

HLA-B35 epitope variants

Eight Gambians with the HLA-B35 antigen were tested using the peptides synthesized to match the HLA-B35 motif (Table 2), and in two individuals CTL were identified. One recognized an epitope from CSP, cp26 (Fig. 3), that represents the 8 amino-terminal residues of the 23 amino-acid peptide previously shown to elicit CTL in three volunteers immunized with irradiated sporozoites¹⁴. In the two volunteers reported to have HLA-B35 this octamer is likely to have been the minimal epitope. Importantly, however, CTL from the Gambian that recognized cp26, did not recognize two other naturally occurring variants of this epitope, cp28 and cp29, and showed only limited recognition of another variant cp27. Conversely, the second Gambian responder recognized the uncommon variant, cp29, but did not recognize cp26, cp27 nor cp28 (Fig. 3). This individual also had CTL specific for an octamer peptide, Is8, from LSA-1.

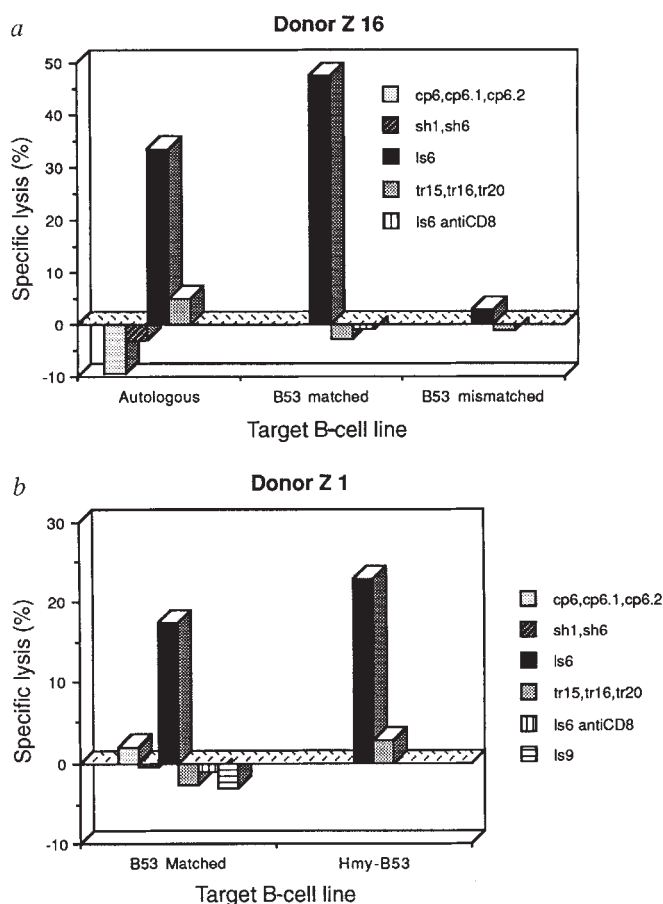
FIG. 2 HLA-B53-restricted CTL. CTL assays using cells from two adult Gambian volunteers with the HLA-B53 antigen, Z 16 (a) and Z 1 (b). Both showed cytolytic activity towards B lymphoblastoid cell lines (BCL) with HLA-B53 when incubated with the Is6 peptide, but not when incubated with the peptides that bound to HLA-B53 from the other three pre-erythrocytic antigens. The cytolytic activity was HLA-B53-restricted and peptide specific. Is9, the octamer peptide derived from Is6 by removing the C-terminal residue was not recognized: Is9 did not bind to HLA-B53 in the assembly assay (Table 2). The B53 matched cell lines were matched to the donors only for HLA-B53; the B53 mismatched line was matched to Z 16 (HLA type A3, A19, B35, B53, Cw3, Cw6) only for HLA-Cw3. The Hmy-B53 line expresses, in significant amounts, only HLA-B53 and -Cw4. CTL activity was inhibitable by an anti-CD8 monoclonal antibody, M236, a gift from R. W. Knowles (Sloane-Kettering, New York) and CTL lines could be maintained by weekly restimulation with peptide-pulsed irradiated autologous B cell lines. One further volunteer from Brefet, out of six HLA-B53 positive individuals tested, showed HLA-B53-restricted CTL responses to the Is6 peptide but none recognized any peptide from the other three antigens. Further samples of blood were obtained after 2–3 weeks from donors Z 16 and Z 1. On this occasion, CTL to Is6 were again grown from Z 1 but not from Z 16. Two caucasian Oxford residents with HLA-B53 but not exposed to malaria were tested as controls and neither responded to any peptide. HLA-B53-positive individuals from the conurbation around the capital Banjul, where malaria transmission is 10–100 times less intense, were also tested. In only one of nine such volunteers could CTL be grown, again only to the Is6 peptide, suggesting that CTL may be more readily detectable in areas of higher malaria transmission. To determine whether the Is6 epitope is conserved or significantly variable among *P. falciparum* isolates in The Gambia, DNA extracted from samples collected from nine children with acute malaria⁵ was examined. A segment of the LSA-1 gene containing the DNA encoding this epitope was amplified by polymerase chain reaction (PCR) and sequenced directly. In all nine samples the previously reported²⁵ 27 base pair (bp) sequence was found.

METHODS. Volunteers from the Gambian village, Brefet, which has hyperendemic malaria, were studied at the time of year with lowest malaria transmission: none had had a recent febrile illness. Peripheral blood lymphocytes were separated from whole blood and incubated at 3×10^6 cells per well of a 24-well Costar plate. The cells were stimulated with peptides that had bound to HLA-B53 in the HLA assembly assay, each at 25 μ M, pooled as follows: cp6, cp6.1, cp6.2, sh1, sh6; and Is6, tr15, tr16 and tr20. The tr16 peptide was included as a non-binding control. At 72 h 10^4 U ml⁻¹ IL-2 (Cetus) was added and CTL assays were done on day 8. ⁵¹Cr-labelled BCL were preincubated for 1 h with 25 μ M of one or more peptides, and without peptide, and a 4 h assay done as described⁶³. Assays (shown here and in Fig. 3) were done at effector to target cell ratios of 50:1 and 8:1 but only the results of the former are shown: lysis at the lower ratio was invariably much lower. CTL lysis of target cells was also detectable without peptide preincubation, by adding the Is6 peptide into the CTL assay at 10^{-7} M (not shown). Background chromium release was from 15–20%. Nonspecific lysis, possibly due to NK-like cells, was higher in Gambians than in Europeans, typically 15–25%. Specific lysis was calculated by the standard formula⁶³ and values in the absence of peptide subtracted to yield the values shown.

Discussion

The identification of CTL to several epitopes in *P. falciparum* antigens indicates that natural exposure to malaria leads to processing of pre-erythrocytic antigens for HLA class I presentation. CD8⁺ CTL, initially identified in unnatural host-parasite combinations in rodents^{11–13,17–19}, were found in volunteers immunized with irradiated sporozoites¹⁴; however, in the latter situation the abnormal termination of liver-stage parasite development may facilitate access of parasite antigens to the hepatocyte cytoplasm^{29–31}. CTL recognizing the conserved Is6 epitope of LSA-1 are restricted through HLA-B53 and, although further work is required to demonstrate CTL in young children and to show that, as in rodent models^{13,17,18}, human CTL can kill intra-hepatic parasites, the findings support our proposal⁵ that the association of this HLA class I antigen with resistance to severe malaria may be mediated by CTL.

Furthermore, although many parasite proteins are expressed in the liver, and we have only examined four for HLA-B53 epitopes, several observations suggest that CTL to Is6 may account for a substantial part of the HLA-B53 protective association. This epitope is predominantly conserved in the parasite



CTL could also be generated by restimulation with the Is6 peptide without tr15, tr16 and tr20 in the medium (not shown). CTL lines were maintained by weekly restimulation with equal numbers of irradiated autologous peptide-pulsed BCL. HLA typing was done by both serological and PCR-based methods as described⁵. The LSA-1 gene was amplified for 35 cycles from nt, 5,372 to nt 5,701 (ref. 25) using the primers GTGCTGAATATGACGATTCA and CCTTTTCCTATTAACTGC, in a buffer with 1 mM MgCl and 10% DMSO, with annealing at 55 °C. The purified amplification products were directly sequenced using a ΔTaq cycle-sequencing kit (USB) and the manufacturers' protocol. Briefly, the reverse strand primer TTCCTTCATCTAAATCATCT (0.5 pmol) was labelled with ³⁵S ATP and the supplied TTP cycle mix and reaction buffer for 80 PCR cycles with annealing at 55 °C for 45 s, and subsequent termination reactions done for 50 cycles with annealing at 60 °C for 45 s.

population in The Gambia, unlike the CSP epitopes identified (ref. 45 and Fig. 3, legend). Moreover, within the hepatocyte the liver-stage parasite develops in a parasitophorous vacuole bounded by an extensive membrane, which probably limits access of parasite antigens to the host cytoplasm and class I presentation pathway^{33,34}. Indeed, access of only a few *P. fal-*

ciparum antigens to class I molecules, and the tendency of CTL responses to be focused on one or few foreign antigens, as observed in some other infections³⁵⁻³⁷, may tend to magnify HLA class I differences in malaria susceptibility: LSA-1 is abundant within the parasitophorous vacuole^{24,38}. Finally, ls6 responses were readily detected using a peptide restimulation

TABLE 2 *P. falciparum* peptides

HLA-B53 peptides																							
	1	2	3	4	5	6	7	8	9	10	pos.		1	2	3	4	5	6	7	8	9	10	pos.
cp1	N	P	N	A	N	P	N	A			150	ls1	I	P	A	I	E	L	P	S	E		1658
cp2	N	P	N	A	N	P	N	A	N		150	ls2	L	P	S	E	N	E	R	G	Y		1663
cp3	K	P	K	H	K	K	L	K	Q		107	ls3	I	P	H	Q	S	S	L	P	Q		1673
cp4	N	P	G	D	G	N	P	D	P	G	115	ls4	L	P	Q	D	N	R	G	N	S		1679
cp5	N	P	D	P	N	A	N	P	N		120	ls5	K	P	E	Q	K	E	D	K	S		1728
cp6	M	P	N	D	P	N	R	N	V		300	ls6	K	P	I	V	Q	Y	D	N	F		1786
cp7	D	P	N	R	N	V	D	G	N		303	ls7	K	P	N	D	K	S	L	Y	D		1850
cp8	S	P	C	S	V	T	C	G	N		347	ls9	K	P	I	V	Q	Y	D	N			1786
cp9	K	P	G	S	A	N	K	P	K		362												
cp10	K	P	K	D	E	L	D	Y	E		368												
cp11	E	P	S	D	K	H	I	E	Q		325												
cp12	E	P	S	D	Q	H	I	E	K		325	tr1	S	P	C	S	V	T	C	G	K		251
cp13	E	P	S	D	K	H	I	K	E		325	tr2	K	P	N	I	P	E	D	S	E	K	361
cp14	E	P	S	D	K	H	I	E	K		325	tr3	I	P	Y	S	P	L	P	P	K		413
cp15	K	P	G	S	A	D	K	P	K		362	tr4	E	P	S	P	N	P	E	E	G	K	327
cp16	K	P	K	D	Q	L	D	Y	A		368	tr5	H	P	E	R	E	E	H	E	K		473
cp17	K	P	K	D	E	L	D	Y	A		368	tr6	N	P	E	N	P	P	N	P	D	I	348
cp18	K	P	K	D	Q	L	D	Y	E		368	tr7	V	P	K	N	P	E	D	D	R		376
cp19	K	P	K	D	Q	L	D	Y			368	tr8	P	P	K	V	L	D	N	E	R		419
cp20	E	P	S	D	Q	H	I	E			325	tr9	V	P	N	S	E	D	R	E	T	R	443
cp21	N	P	D	P	N	A	N	P	N	V	120	tr10	R	P	H	G	R	N	N	E	N	R	452
cp22	N	P	N	V	D	P	N	A	N		126	tr11	E	P	E	D	D	Q	P	R	P	R	297
cp23	D	P	N	A	N	P	N	V	D		130	tr12	L	P	P	K	V	L	D	N	E	R	418
cp6.1	M	P	N	Y	P	N	R	N	V		300	tr13	I	P	D	S	I	Q	D	S	L		164
cp6.2	M	P	N	N	P	N	R	N	V		300	tr14	I	P	E	D	S	E	K	E	V		364
												tr15	E	P	A	P	F	D	E	T	L		529
sh1	I	P	S	L	A	L	M	L	I		7	tr16	V	P	D	E	P	E	D	D	Q		295
sh2	K	P	A	G	K	G	S	P	S		33	tr18	I	P	K	K	P	E	N	K	H		390
sh3	S	P	S	T	L	Q	T	P	G		39	tr19	T	P	K	H	P	E	R	E	E		470
sh4	T	P	G	S	S	S	G	A	S		45	tr20	H	P	S	D	G	K	C	N	L		206
sh5	G	P	N	Q	G	G	L	S	Q		58	tr21	G	P	F	M	K	A	V	C	V		228
sh6	M	P	L	E	T	Q	L	A	I		77	tr22	P	P	K	W	E	P	L	D	V		287
HLA-B35 peptides																							
	1	2	3	4	5	6	7	8	9	10	pos.		1	2	3	4	5	6	7	8	9	10	pos.
cp26	K	P	K	D	E	L	D	Y			368	ls2	L	P	S	E	N	E	R	G	Y		1663
cp27	K	P	K	D	Q	L	D	Y			368	ls8	K	P	N	D	K	S	L	Y			1850
cp28	K	P	K	D	Q	L	N	Y			368												
cp29	K	S	K	D	E	L	D	Y			368												
cp30	E	P	S	D	K	H	I	E	Q	Y	325	tr24	H	P	S	D	G	K	C	N	L	Y	206
cp31	E	P	S	D	Q	H	I	E	K	Y	325	tr25	V	P	G	A	A	T	P	Y			519

Antigens studied: Peptides were identified in the four sequenced pre-erythrocytic antigens of *P. falciparum*: (1) circumsporozoite protein²⁰ (CSP), a 412 amino-acid sporozoite protein; (2) thrombospondin-related anonymous protein²¹ (TRAP), 559 amino acids and expressed by sporozoites²² and blood-stage parasites; (3) liver-stage-specific antigen-1 (ref. 24, 25), 1,909 amino acids and expressed only by the liver-stage parasite; and (4) sporozoite hepatocyte-binding antigen²³ (SHEBA or Pfs16), 157 amino acids and found in both sporozoites and gametocytes. Antigens expressed by sporozoites will also be present during the early phase of hepatocyte development. CSP and LSA-1 have large central repeats spanning 164 and 1,475 amino acids, respectively^{20,25}. **Top:** Peptides synthesized and tested in the HLA assembly assay for binding to HLA-B53. Peptides shown to bind to HLA-B53 are highlighted in bold type. The amino-acid sequence, using the single-letter amino-acid code, and the number of amino-terminal residue in the published sequence^{20,21,23,25} is shown. The CSP, LSA-1, TRAP and SHEBA antigens are indicated by the prefixes cp, ls, tr and sh, respectively. All possible nonamer peptides with proline at position 2 were synthesized for CSP, LSA-1 and SHEBA; where a sequence had been shown to be polymorphic variant peptides, for example cp10, cp16-18, were also synthesized. The peptides from CSP which bound to HLA-B53, that is, cp6, cp6.1 and cp6.2 are allelic variants⁵⁷. Additionally, some octamer and decamer peptides were analysed but none of these bound. The decamer, cp4, was made with an added terminal glycine to aid synthesis. Inspection of the sequences of peptides binding to HLA-B53 indicated that several branched aliphatic or aromatic residues, but no polar or hydrophilic residues, can be accommodated at the C terminus. So, for TRAP which has a total of 48 prolines, a subset of 21 of these, including all possible peptides with proline at position 2 and C termini of residues, V, A, L, I, F or W were synthesized. The cell line T2-B53 has endogenous HLA-A2 and HLA-B51 genes as well as the transfected HLA-B53 gene. HLA-B51, as well as HLA-B53, is immunoprecipitated by the antibodies used, so data on peptide binding to this HLA type was obtained in the same experiments: only peptides sh1, sh6 and cp6, but not cp6.1 nor cp6.2, bound to HLA-B51. All the highlighted peptides as well as the eluted self-peptide YPAEITLTW, and an HIV-2 HLA-B53 epitope, TPYDINQML (our unpublished data) were found to show 50% maximum binding to HLA-B53 in the micromolar range (2-6 μ M) (Fig. 1). **Bottom:** Peptides synthesized to match the HLA-B35 motif. Only a single nonamer peptide in the four pre-erythrocytic antigens, ls2, has proline at position 2 and tyrosine at position 9. So, all possible peptides with proline at 2 and tyrosine at 8 or 10, as well as their allelic variants, were also tested. In addition to the 10 peptides shown two longer peptides, cp24 and cp25, ENDIEKKICKMEKCS and ELDYANDIEKKICKM, were also included in CTL assays: these, with cp26, overlap the 23-mer sequence previously shown to contain a CTL epitope¹⁴. Peptides shown to be epitopes (Fig. 3) are highlighted in bold type.

method that appears less efficient at growing CTL than conventional viral restimulation methods (ref. 39 and our unpublished data).

We could not grow CTL to Is6 from all B53-positive donors during the low transmission season for malaria but, as previous work suggested¹⁵, detectable CTL to *P. falciparum* may be relatively short-lived in the absence of boosting, and low precursor frequencies may be common because of the very small numbers of hepatocytes infected by each sporozoite inoculum⁴⁰. Even in immune adults CTL do not completely prevent bloodstage infection. But this natural partially effective response might be enhanced by immunization, and recently a bacterial CTL epitope identified in mice using a class I motif has been shown to induce a protective response after immunization^{41,42}. More generally, our identification of a likely mechanism for an HLA-disease association by characterization of peptides bound to the relevant HLA type and screening of candidate antigens for epitopes congruent with a derived motif, should encourage similar approaches to other disease associations.

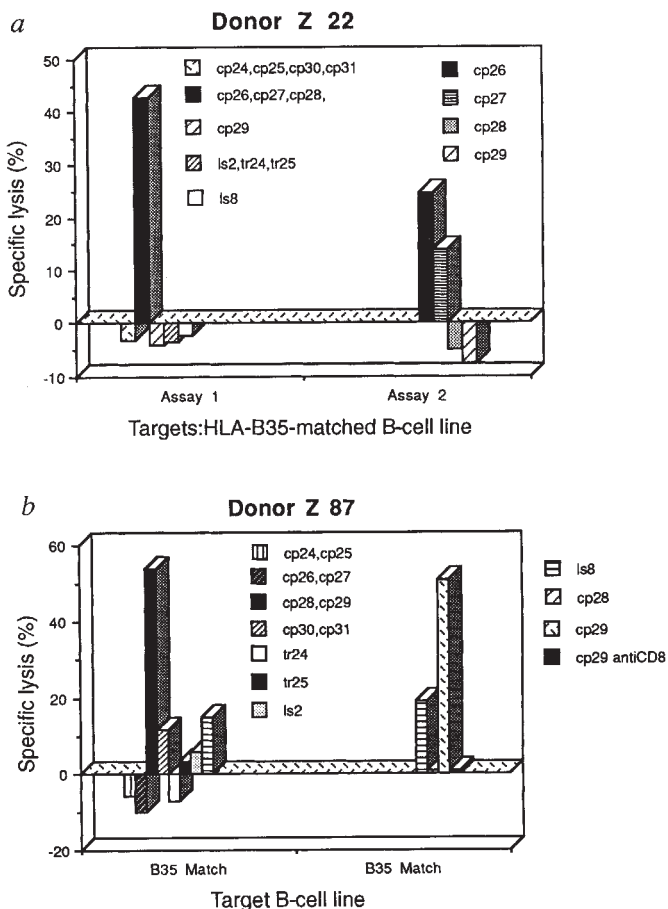
Polymorphism clustering in the T-cell epitopes of the CSP generated debate about the origin and maintenance of this variation and raised concerns about its possible influence on vaccine efficacy⁴³⁻⁴⁸. The previously identified 23 amino-acid CTL epitope¹⁴ included polymorphic residues, and three of these are contained in the minimal HLA-B35 restricted octamer epitope, with variant epitopes escaping CTL recognition. Hence,

FIG. 3 HLA-B35-restricted CTL. CTL assay results from donor Z 22 (a) and donor Z 87 (b), both residents of the same village in The Gambia as donors Z 16 and Z 1 (Fig. 2). Cells from donor Z 22 were initially tested (assay 1) on five different sets of (1-4) peptides as shown, using as targets a BCL HLA-matched to the donor only for HLA-B35. Cells preincubated with the peptides cp26, cp27 and cp28 were lysed. In assay 2, these peptides and cp29, which are four allelic variants (Table 2), were tested individually, again at an effector to target cell ratio of 50:1. Cp26 was recognized, 24.4% specific lysis, and cp28 and 29 were not: cp27 showed 13.7% specific lysis, just below the level we regard as positive, 15%. Cells from donor Z 87 were tested initially on HLA-B35-matched BCL pre-incubated with seven different peptides, as shown. Significant lysis was found for the cp28, cp29 mixture (54%) and for Is8 (15%); the weak response to cp30 and cp31 was not repeatable. A further assay, using a different BCL again matched only for HLA-B35, confirmed the Is8 result and showed that cp29 but not cp28 was the CSP peptide recognized. CTL from Z 87 also lysed autologous BCL targets preincubated with cp29 and Is8 (not shown). CTL lysis of cp29-pulsed BCL was blocked by the anti-CD8 monoclonal antibody, M236. Using a further sample from donor Z 87, fresh (unrestimulated) PBL were used in a CTL assay on cp29-pulsed targets but no lysis was observed. In all, eight donors with HLA-B35 were tested, all from the village of Brefet, and only these two responded to any of the peptides tested. PBL from two Oxford residents with HLA-B35 were restimulated with all 12 peptides and grew no CTL. All three HLA-B35 epitopes identified here are octamers, with several shared residues, even though most eluted self peptides were nonamers (Table 1). The available data suggest that responses to Is8 in HLA-B35 individuals may be less frequent than Is6 responses in HLA-B53 individuals but larger numbers are required to substantiate this. To estimate the relative frequencies of the four allelic variants cp26-cp29 in *P. falciparum* in The Gambia, DNA was analysed from samples collected from 156 children with acute malaria⁵. A segment of the CSP gene containing the DNA encoding this epitope was amplified by PCR and the allelic variants detected by specific oligonucleotide probes. More than one parasite variant (mean 2.0) can frequently be detected in these children with acute malaria⁶⁴. The frequencies of the four variants were: cp26, 45%; cp27, 78%; cp28, 26%; cp29, 11%. The single amino-acid changes between some of these peptides is sufficient to abolish CTL recognition, as shown in other systems^{65,66}. The poor or absent recognition of cp27 and cp28 may result from the charge change at position 5 of the peptide preventing binding to HLA-B35. The alternative, that neither individual had been infected with a parasite carrying these commoner variants, seems less likely.

METHODS. Peptide stimulations and CTL assays were done essentially as described in the legend to Fig. 2. PBL were incubated with peptides each at 25 μ M, pooled as follows: pool 1, the eight CSP peptides cp24-cp31; pool 2, Is2, Is8, tr24 and tr25. All HLA-B35 individuals were typed by the previously described PCR method⁵ to confirm that they had the B*3501 rather than the B*3502 or B*3503 subtypes: these latter subtypes are not amplified

CTL to this epitope generated by one parasite strain will not protect against many other strains. Although CSP epitope variation may be limited in some regions^{49,50}, the extensive polymorphism seen in Africa will be difficult to encompass in multi-valent vaccines. Conversely, the observation that the CSP CTL epitope contains only non-synonymous nucleotide changes supports the proposal that much of the observed CSP sequence variation has been generated and maintained by CTL selection pressure^{43,44,51}, the intensity of which may be frequency-dependent. This indication of parasite adaptation to avoid host CTL responses provides evidence of a different type for a protective role for CTL in *P. falciparum* malaria. The contrasting conservation of the Is6 epitope might indicate an important functional role for this sequence that prevents escape mutants becoming prevalent.

CTL against LSA-1, identified here in Africans naturally exposed to malaria, may also be important in response to immunization with irradiated sporozoites. To produce immunity, live sporozoites must be injected intravenously after an irradiation dosage that allows limited intra-hepatic development, suggesting that expression of early liver-stage antigens is required for protection. But all reports of CTL in rodents and humans¹¹⁻¹⁹ as well as most pre-erythrocytic vaccine research have focused on sporozoite antigens, particularly CSP, even though CTL to CSP alone appear insufficient for protection against *P. falciparum*¹⁴.



by the primers used. To identify alleles of the CSP gene by PCR, primers AATGCTAATGCCAACAATGCTG and CGACATTAAACACACTGGAAC (compare with ref. 45) were used (in 1.5 mM MgCl₂) with annealing at 58 °C. Amplification products were fixed to nitrocellulose filters and alleles^{45,47} detected using the following probes with washing in 6 × SSC at the stated temperature: cp26, ACGAATTAGATTATGCAA and ACGAATTAGATTATGAAA, 46 °C and 44 °C; cp27, ACCAATTAGATTATGCAA, 46 °C; cp28, ACCAAT-TAAATTATGAAA, 42 °C; cp29, TAAATCTAAAGACGA, 38 °C.

The association of HLA-B53 with malaria resistance and the observation that HLA-B53-restricted CTL recognize only LSA-1 of several major pre-erythrocytic antigens from *P. falciparum*, constitute a genetic approach to implicating this antigen in

providing protective immunity to malaria, and encourage further assessment of LSA-1 as a component of future subunit vaccines. □

Received 20 August; accepted 27 October 1992.

- Amiel, J. L. in *Histocompatibility Testing 1967* 79–83 (Munksgaard, Copenhagen, 1967).
- Tiwari, J. L. & Terasaki, P. I. (eds) *HLA and Disease Associations* (Springer, New York, 1985).
- Falk, K., Rotzko, O., Stevanovic, S., Jung, G. & Rammensee, H. *Nature* **351**, 290–296 (1991).
- Jardetsky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R. & Wiley, D. C. *Nature* **353**, 326–329 (1991).
- Hill, A. V. S. *et al. Nature* **352**, 595–600 (1991).
- Perlmann, P. & Miller, L. (eds) *Immun. Lett.* **25**, 1–294 (1990).
- Nussenzeig, R. S., Vanderberg, J., Most, H. & Orton, C. *Nature* **222**, 488–489 (1969).
- Clyde, D. F., McCarthy, V. C., Miller, R. M. & Hormick, R. B. *Am. J. med. Sci.* **266**, 398–403 (1973).
- Schofield, L. *et al. Nature* **330**, 664–666 (1987).
- Weiss, W. R., Sedegah, M., Beaudoin, R. L., Miller, L. H. & Good, M. F. *Proc. natn. Acad. Sci. U.S.A.* **85**, 573–576 (1988).
- Kumar, S. *et al. Nature* **334**, 258–260 (1988).
- Romero, P., Maryanski, J. L., Corradin, G., Nussenzeig, R. S. & Nussenzeig, V. *Nature* **341**, 323–326 (1989).
- Hoffman, S. L. *et al. Science* **244**, 1078–1081 (1989).
- Malik, A., Egan, J. E., Houghten, R. A., Sadoff, J. C. & Hoffmann, S. L. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3300–3304 (1991).
- Doolan, D. L., Houghten, R. A. & Good, M. F. *Int. Immun.* **3**, 511–516 (1991).
- Sedegah, M. *et al. J. Immun.* **149**, 966–971 (1992).
- Weiss, W. R. *et al. J. exp. Med.* **171**, 1083–1090 (1990).
- Rodrigues, M. M. *et al. Int. Immun.* **3**, 579–585 (1991).
- Khusmith, S. *et al. Science* **252**, 715–718 (1991).
- Dame, J. B. *et al. Science* **225**, 593–599 (1984).
- Robson, K. J. H. *et al. Nature* **335**, 79–82 (1988).
- Cowan, G., Krishna, S., Crisanti, A. & Robson, K. J. H. *Lancet* **339**, 1412–1413 (1992).
- Moelans, I. I. M. D., Meis, J. F. G. M., Koonen, C., Konings, R. N. H. & Schoenmakers, J. G. G. *Molec. Biochem. Parasitol.* **45**, 193–204 (1991).
- Guerin-Marchand, C. *et al. Nature* **329**, 164–167 (1987).
- Zhu, J. & Hollingdale, M. *Molec. Biochem. Parasitol.* **48**, 223–226 (1991).
- Elvin, J., Cerundolo, V., Elliot, T. & Townsend, A. *Eur. J. Immun.* **21**, 2025–2031 (1991).
- Madden, D. R., Gorga, J. C., Strominger, J. L. & Wiley, D. C. *Nature* **353**, 321–325 (1991).
- Harris, R. & Zervas, J. D. *Nature* **221**, 1062–1063 (1969).
- Druhlle, P. & Marchand, C. in *New Strategies in Parasitology* (ed. McAdam, K. P. W. J.) (Churchill-Livingstone, Edinburgh, 1989).
- Mellouk, S., Lunel, F., Sedegah, M., Beaudoin, R.-L. & Druhlle, P. *Lancet* **335**, 721 (1990).
- Suhrbier, A., Winger, L. A., Castellano, E. & Sinden, R. E. *Infect. Immun.* **58**, 2834–2839 (1990).
- McGregor, I. A. & Wilson, R. J. M. in *Malaria: Principles and Practice of Malariaology* 559–620 (Churchill-Livingstone, Edinburgh, 1988).
- Meis, J. F. G. M. *et al. Cell Tiss. Res.* **244**, 345–350 (1986).
- Suhrbier, A. *Parasitol. Today* **7**, 160–163 (1991).
- Van Bleek, G. M. & Nathanson, S. G. *Nature* **348**, 213–216 (1990).
- Jacobsen, S., Shida, H., McFarlin, D. E., Fauci, A. S. & Koenig, S. *Nature* **348**, 245–248 (1990).
- Nixon, D. F., Broliden, K., Ogg, G. & Broliden, P. A. *Immunology* **76**, 515–534 (1992).
- Hollingdale, M. R. *et al. Immun. Lett.* **25**, 71–81 (1990).
- Carreno, B. M., Turner, R. V., Biddison, W. E. & Coligan, J. E. *J. Immun.* **148**, 894–899 (1992).
- Rosenberg, R., Wirtz, R. A., Schneider, I. & Burge, R. *Trans. R. Soc. trop. Med. Hyg.* **84**, 209–212 (1990).
- Pamer, E. G., Harty, J. T. & Bevan, M. J. *Nature* **353**, 852–855 (1991).
- Harty, J. T. & Bevan, M. J. *J. exp. Med.* **175**, 1531–1538 (1992).
- De la Cruz, V. F., Lal, A. A. & McCutchan, T. F. *J. biol. Chem.* **262**, 11935–11939 (1987).
- Good, M. F., Kumar, S. & Miller, L. H. *Immun. Today* **9**, 351–355 (1988).
- Lockyer, M. J., Marsh, K. & Newbold, C. I. *Molec. Biochem. Parasitol.* **37**, 275–280 (1989).
- Arnot, D. *Parasitol. Today* **5**, 138–142 (1989).
- Good, M. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 1199–1203 (1988).
- Lockyer, M. *Parasitol. Today* **6**, 76 (1990).
- Yoshida, N. *et al. Expl Parasitol.* **71**, 386–392 (1990).
- Doolan, D. L., Saul, A. J. & Good, M. F. *Infect. Immun.* **60**, 765–682 (1991).
- McCutchan, T. F. & Waters, A. P. *Immun. Lett.* **25**, 23–26 (1990).
- Allsopp, C. E. M. *et al. Hum. Immun.* **30**, 105–109 (1991).
- Hayashi, H. *et al. Immunogenetics* **32**, 195–199 (1990).
- Sutton, J. *et al. Eur. J. Immun.* (in the press).
- Yamamoto, J., Kariyone, A., Akiyama, N., Kano, K. & Takiguchi, M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 2583–2587 (1990).
- Barnstable, C. J. *et al. Cell* **14**, 9–20 (1978).
- Doolan, D. L., Saul, A. J. & Good, M. F. *Infect. Immun.* **60**, 675–682 (1992).
- Townsend, A. *et al. Cell* **62**, 285–295 (1990).
- Schumacher, T. N. M. *et al. Cell* **62**, 563–567 (1990).
- Cerundolo, V. *Nature* **345**, 449–456 (1990).
- Elvin, J. *et al. J. Immun. Meth.* (in the press).
- Ellis, S. *et al. Hum. Immun.* **13**, 13–20 (1985).
- Bodmer, H. C., Gotch, F. M. & McMichael, A. J. *Nature* **337**, 653–655 (1989).
- Conway, D., Greenwood, B. M. & McBride, J. S. *Parasitology* **103**, 1–6 (1991).
- Pircher, H. P. *et al. Nature* **346**, 629–633 (1990).
- Phillips, R. E. *et al. Nature* **354**, 453–459 (1991).

ACKNOWLEDGEMENTS. We thank the volunteers for blood samples, M. Sanyang, A. B. Hill, S. Srikumar, T. Elliot, S. Rowland-Jones and S. N. R. Yates for assistance and advice, K. J. H. Robson, B. Askonas, D. Kwiatkowski, C. I. Newbold, M. R. Hollingdale and R. E. Sinden for discussions, K. Reid for facilities, J. Bodmer, T. Seddon, D. Watling and C. Dixon for W6/32 and Cetus Corporation for recombinant IL-2. A.V.S.H. is a Wellcome Trust Senior Clinical Fellow.

LETTERS TO NATURE

Astrophysical ${}^7\text{Li}$ as a product of Big Bang nucleosynthesis and galactic cosmic-ray spallation

Keith A. Olive* & David N. Schramm†

* School of Physics and Astronomy, University of Minnesota, Minneapolis, Minnesota 55455, USA

† University of Chicago, Chicago, Illinois 60637, USA

RECENTLY measured abundances of beryllium^{1–4} and boron⁵ in a number of hot population II halo stars are orders of magnitude above the predicted abundances of those elements from standard Big Bang nucleosynthesis⁶. Be and B do not, however, show a plateau of constant abundance over a wide range of low metallicities and high temperatures, as is the case for ${}^7\text{Li}$ (refs 7–15). The implication is that the ${}^7\text{Li}$ abundance is largely primordial, whereas the Be and B abundances are due to galactic cosmic ray (GCR) spallation reactions^{16–22} on top of a much smaller Big Bang component²³. But GCR spallation should also produce ${}^7\text{Li}$. As a consistency check on the combination of Big Bang nucleosynthesis and GCR spallation, we use the Be and B data to subtract from the measured ${}^7\text{Li}$ abundance an estimate of the amount generated by GCR spallation^{21,22} for each star in the sample, and then add to this baseline an estimate of the metallicity-dependent augmentation of ${}^7\text{Li}$, due to spallation. The slightly reduced primordial ${}^7\text{Li}$ abundance is still consistent with Big Bang

nucleosynthesis, and a single GCR spallation model can fit the Be, B and corrected ${}^7\text{Li}$ abundances for all the stars in the sample.

The dominant product of Big Bang nucleosynthesis⁶ is ${}^4\text{He}$, which is produced with a relative abundance by mass of $Y \approx 0.24$ (leaving a primordial hydrogen mass fraction of $\sim 76\%$). ${}^4\text{He}$ is accompanied by lesser amounts of D and ${}^3\text{He}$, ($\text{D}, {}^3\text{He})/\text{H} \approx 10^{-5}$ (by number). In contrast, ${}^7\text{Li}$ is produced with even lower abundances (${}^7\text{Li}/\text{H} \approx 10^{-10}$ in the standard model). The fact that the predicted abundances of these light element isotopes can be explained over this range of nearly 10 orders of magnitude is clearly a success for the simplest nucleosynthesis model. Big Bang nucleosynthesis calculations can also predict the primordial abundances of other light element isotopes²³, namely ${}^6\text{Li}$, ${}^9\text{Be}$, ${}^{10}\text{B}$ and ${}^{11}\text{B}$. These isotopes are produced in much smaller quantities (${}^6\text{Li}/\text{H} \approx 10^{-14}$, ${}^9\text{Be}/\text{H} \approx 10^{-18}$, ${}^{10}\text{B}/\text{H} \approx 10^{-20}$, ${}^{11}\text{B}/\text{H} \approx 10^{-18}$, for a baryon to photon ratio of $\eta \approx 3 \times 10^{-10}$ (the value required to maintain consistency with the light element abundances).

Since the observation of the ${}^7\text{Li}$ plateau^{7–9}, it has been argued that the plateau value of (${}^7\text{Li}/\text{H}) \approx 10^{-10}$ corresponds to primordial ${}^7\text{Li}$. Hence, ${}^7\text{Li}$ has been a key element in tests of consistency for Big Bang nucleosynthesis. The stars making up the plateau (there are nearly 40 of them now) are all population II halo dwarfs. They have low metallicity, $[\text{Fe}/\text{H}] \approx -1.3$ ($[\text{X}/\text{H}]$ corresponds to the log abundance by number relative to the solar value), with some going as low as $[\text{Fe}/\text{H}] = -3.5$. They also have high surface temperatures $T > 5,500$ K. Cooler halo stars are observed to have considerably less ${}^7\text{Li}$, confirming stellar models that depict convective depletion at $T \approx 5,500$ K (see for

example ref. 25). Over the entire range ($5,500 < T < 6,300$ K and $-3.5 \leq [\text{Fe}/\text{H}] \leq -1.3$) the ${}^7\text{Li}$ abundance is remarkably constant. The plateau stars have a well determined mean

$$[{}^7\text{Li}] = 2.08 \pm 0.02 \quad (1)$$

(where we use the astronomical convention $[{}^7\text{Li}] = 12 + [{}^7\text{Li}/\text{H}]$) and show a dispersion of ~ 0.12 which is consistent with the observational uncertainty in the measurements. We stress that in equation (1) we have only quoted a statistical error and have not included any systematic errors, which may be sizeable (~ 0.1) and could arise, for example, from the methods of determining the temperatures of these stars. Unfortunately the literature does not supply all the information needed to assess more the systematic errors accurately. Below, all our errors quoted are only statistical; thus the true error is always larger. Furthermore, there are no significant departures from constancy in $[\text{Li}]$ in these stars. The dispersion from the mean value, given as χ^2 per degree of freedom, is $\chi^2 = 1.26$. There is no correlation with metallicity: $[\text{Li}] = 2.11 \pm 0.09 + (0.02 \pm 0.04) [\text{Fe}/\text{H}]$, $\chi^2 = 1.26$, $r = -0.056$. The small value of the correlation coefficient, r , shows that the faint hint of a correlation with temperature is not significant: $[\text{Li}] = 0.45 \pm 0.62 + (0.0003 \pm 0.0001)T$, $\chi^2 = 1.11$, $r = 0.390$, or $d(\text{Li}/\text{H})/dT = 3.6 \times 10^{-14}$, in good agreement with the value given by Hobbs and Thorburn¹⁵. This lack of trend (the small slope with respect to T leads to a change in Li which is smaller than the error in Li over the entire temperature range) has lent strong credence to the assumption that population II lithium has a primordial origin.

Several halo dwarfs have been shown to have a measurable ${}^9\text{Be}$ abundance¹⁻⁴ (most of these are in the ${}^7\text{Li}$ data set). Unlike ${}^7\text{Li}$, the ${}^9\text{Be}$ abundance is strongly correlated with metallicity

$$[{}^9\text{Be}] = (-10.19 \pm 0.54) + (1.13 \pm 0.27)[\text{Fe}/\text{H}] \quad (2)$$

for which $\chi^2 = 0.28$, $r = 0.93$. Thus it is clear that the observed ${}^9\text{Be}$ is not primordial. As the standard model prediction for primordial²³ ${}^9\text{Be}/\text{H}$ is $\sim 10^{-18}$ – 10^{-17} whereas the observed abundances are 10^{-13} – 10^{-12} , the strong correlation is not really a surprise. Unless a ${}^9\text{Be}$ plateau can be established, the measured ${}^9\text{Be}$ abundances on their own say very little about standard or even nonstandard nucleosynthesis, as no primordial value can be determined. Unlike the case for ${}^4\text{He}$, where the primordial component is dominant and one can determine a definite primordial value by extrapolation to near-zero metallicity, the primordial component of ${}^9\text{Be}$ is presumably negligible compared with the observed ${}^9\text{Be}$.

TABLE 1 Observed Be and Li and extracted Big Bang abundance of ${}^7\text{Li}$

Star	[Fe/H]	(${}^7\text{Li}/\text{Be}$) _{GCR}	[Be] _{obs}	[Li] _{obs}	[Li] _{BB}
HD16031	-1.9	80 ± 30	-0.37 ± 0.25	2.03 ± 0.20	1.86 ± 0.32
HD134169	-1.2	11 ± 2	+0.65 ± 0.4	2.21 ± 0.09	2.06 ± 0.22
HD140283	-2.6	234 ± 14	-1.04 ± 0.19	2.09 ± 0.07	2.01 ± 0.09
HD160617	-1.9	38 ± 9	-0.47 ± 0.18	2.20 ± 0.20	2.16 ± 0.22
HD189558	-1.3	26 ± 8	+0.00 ± 0.4	2.04 ± 0.20	1.92 ± 0.30
HD201891	-1.3	12 ± 4	+0.40 ± 0.4	1.98 ± 0.07	1.82 ± 0.22
HD213617	-2.2	161 ± 61	-0.65 ± 0.25	2.17	2.05 ± 0.28
BD23° 3912	-1.5	43 ± 16	+0.30 ± 0.4	2.36 ± 0.10	2.16 ± 0.30

For completeness, we note that there are new observations⁵ of B in three halo dwarfs. Again there is a strong correlation with metallicity

$$[\text{B}] = (-8.48 \pm 0.67) + (1.42 \pm 0.33)[\text{Fe}/\text{H}] \quad (3)$$

with $\chi^2 = 0.27$, $r = 0.99$. And as for Be, the measured abundances are orders of magnitude above the Big Bang yields.

These new observations are relevant to our understanding of GCR spallation processes¹⁶⁻²⁰: p, α on ${}^4\text{He}$, C, N and O. Here, we make the simple set of assumptions used in refs 21 and 22 (the reader is referred there for details). It was found that the abundances of [Be] and [B] are linearly correlated with [Fe]; $[{}^7\text{Li}]$ is less strongly correlated as much of the ${}^7\text{Li}$ is produced by $\alpha + \alpha$ collisions rather than by spallation on CNO; spallation produces ${}^6\text{Li}/{}^7\text{Li} \approx 0.9$ and predicts $\text{B}/\text{Be} \approx 7$ – 17 , with the simplest model yielding $\text{B}/\text{Be} \approx 12$ – 14.5 , which is within 1σ of the observation⁵ ~ 10 . As ${}^6\text{Li}$ is depleted much more rapidly than ${}^7\text{Li}$ and Be, observation of ${}^6\text{Li}$ in halo dwarfs might only be possible in extreme high-temperature ($T > 6,200$ K) dwarfs^{25,26}, and then only if Li is not depleted by mass loss or diffusion²⁷. From the new observation²⁴ of ${}^6\text{Li}$ in the halo subdwarf HD84927 with ${}^6\text{Li}/({}^6\text{Li} + {}^7\text{Li}) \approx 0.05 \pm 0.02$ at $T \approx 6,200$ K, and because of the fragility²⁶ of ${}^6\text{Li}$, one can therefore conclude that the ${}^7\text{Li}$ observed⁷⁻¹⁰ in this star $[\text{Li}] = 2.11 \pm 0.07$ is indeed not significantly depleted. As we will see below, this observation has important consequences for nonstandard models.

The consistency of Big Bang nucleosynthesis with the Be and Li abundances has been examined²². It was shown that although a sizeable fraction of the total ${}^7\text{Li}$ may be produced, the observed ${}^7\text{Li}$ is consistent with the prediction of GCR spallation and a primordial value of $[\text{Li}] = 2.00$ – 2.12 . One can go further than the consistency check of Walker *et al.*²² by systematically

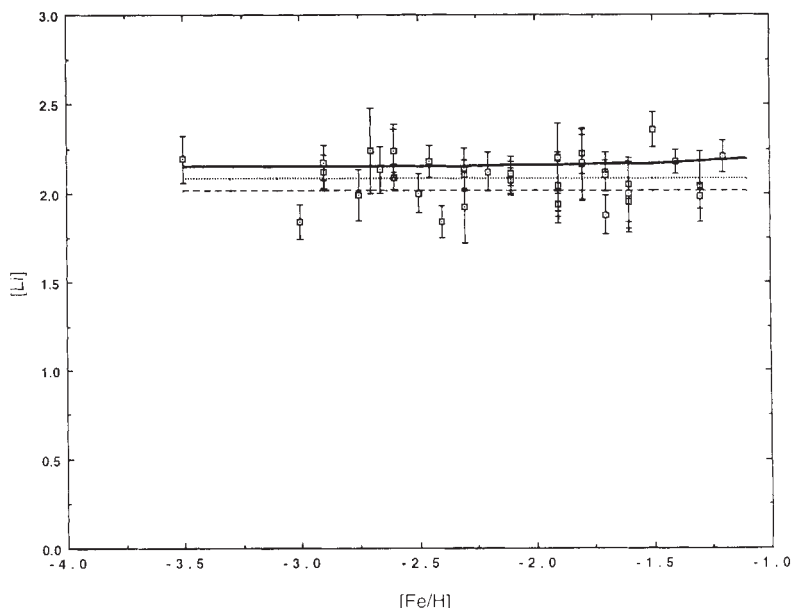


FIG. 1 The ${}^7\text{Li}$ data as a function of the $[\text{Fe}/\text{H}]$. The dotted line corresponds to the mean of the data $[\text{Li}] = 2.08$, $\chi^2 = 1.26$. The dashed line corresponds to the extracted primordial value of $[\text{Li}] = 2.01$. The solid curve corresponds to the sum of the primordial value and the metallicity-dependent GCR-spallation produced ${}^7\text{Li}$ with $\chi^2 = 1.75$.

subtracting out the ${}^7\text{Li}$ produced by GCR spallation from the observations. Of the 12 stars with observed ${}^9\text{Be}$, 10 have measured ${}^7\text{Li}$. Of these 10 we omit HD76932, which is usually omitted from the ${}^7\text{Li}$ plateau data set because of its high metallicity, and HD34328 which is reported⁴ to be a dubious measurement (because of an uncertain spectrographic setting). This leaves us with eight stars from which we attempt to determine the primordial ${}^7\text{Li}$ abundance. These stars are listed in Table 1 along with $[\text{Fe}/\text{H}]$ (an unweighted mean of measured values), the GCR-spallation ratio ${}^7\text{Li}/{}^9\text{Be}$, the observed ${}^9\text{Be}$ and ${}^7\text{Li}$ abundances and the derived primordial abundances from

$$({}^7\text{Li}/\text{H})_{\text{BB}} = ({}^7\text{Li}/\text{H})_{\text{obs}} - ({}^7\text{Li}/{}^9\text{Be})_{\text{GCR}} ({}^9\text{Be}/\text{H})_{\text{obs}} \quad (4)$$

Where it is available, we use the weighted average of observed C, N and O in these stars, otherwise we use the simple starting point given above in ref. 22 for population II stars. Observational errors in all measurable quantities have been propagated leading to the larger errors for $[{}^7\text{Li}]_{\text{BB}}$ shown in Table 1. The weighted mean (again ignoring possible systematics) for these eight stars is

$$[\text{Li}]_{\text{BB}} = 2.01 \pm 0.07 \quad (5)$$

Because of the greater uncertainty, the 2σ upper limit is essentially the same as one would obtain from equation (1). The dispersion in observed ${}^7\text{Li}$ of these eight stars is given by a χ^2 per degree of freedom of 1.73, although because of the larger uncertainties the χ^2 per degree of freedom of $[\text{Li}]_{\text{BB}}$ is small, 0.26. The extracted abundance of Li (equation (5)) is not too low to be consistent with standard Big Bang nucleosynthesis.

In principle one can repeat this exercise with the B data shown in Table 2. The implied primordial abundance of ${}^7\text{Li}$ from the B data (by an analogous procedure) is also $[\text{Li}] = 2.01 \pm 0.06$, in perfect (coincidental) agreement with the value derived from Be. We are also encouraged that the B/Be ratios are consistent with the GCR predictions²².

Given a primordial value of $[\text{Li}] = 2.01$, we can now determine the ${}^7\text{Li}$ abundance as a function of $[\text{Fe}/\text{H}]$, using $[\text{C}/\text{H}] = [\text{N}/\text{H}] = [\text{Fe}/\text{H}]$ and $[\text{O}/\text{Fe}] = 0.5$ so that²²

$$[\text{Li}]_{\text{GCR}} = 1.59 + \log(1 + 4.53 \times 10^{[\text{Fe}/\text{H}]}) \quad (6)$$

and

$$(\text{Li}/\text{H})_{\text{total}} = (\text{Li}/\text{H})_{\text{BB}} + (\text{Li}/\text{H})_{\text{GCR}} \quad (7)$$

(The normalization has been fixed by $({}^9\text{Be}/\text{H})_{\text{OBS}} = ({}^9\text{Be})_{\text{GCR}}$ which fixed the exposure time $\Delta t = 10.7$ Gyr.) $[\text{Li}]$ total is plotted as a function of $[\text{Fe}/\text{H}]$ in Fig. 1 along with the baseline $[\text{Li}]_{\text{BB}} = 2.01$ (dashed) the ${}^7\text{Li}$ data set and the mean of the data $[\text{Li}] = 2.08$ (dotted). Given the uncertainties in the measurements and calculations, one cannot identify a discrepancy between the data and the model prediction (solid curve). We are not suggesting that the solid curve in Fig. 1 (from equation (7)) gives a better fit to the data. But one can clearly see that the extracted primordial abundance (equation (5)) and the predicted total abundance of ${}^7\text{Li}$ (equations (6), (7)) can be used to argue for the consistency of the Big Bang and GCR production of ${}^7\text{Li}$. The χ^2 per degree of freedom is now 1.75. It could be even lower if we make the correction suggested by Hobbs and Thorburn¹⁵ on the left-most discrepant star G238-30. They use a higher surface temperature leading to $[\text{Li}] = 2.05$ rather than $[\text{Li}] = 1.84$ for the same equivalent width. This correction would reduce χ^2 to 1.52. We are not aware of any corrections suggested for the other two discrepant stars. One should note that we have neglected the effects of diffusion (a point we will return to shortly). The models of Deliyannis *et al.*²⁵ using standard Li isochrones are best fitted with an initial abundance of $[\text{Li}] = 2.17$, close to the model prediction of $[\text{Li}] \approx 2.15$. GCR models with flatter spectra yield smaller GCR Li corrections for given Be and B abundances.

Finally, we consider the implications of these results for inhomogeneous nucleosynthesis²⁸⁻³⁰ and extreme depletion in

TABLE 2 Observed B and Li and extracted Big Bang abundance of ${}^2\text{Li}$

Star	$[\text{Fe}/\text{H}]$	$({}^7\text{Li}/\text{B})_{\text{GCR}}$	$[\text{B}]_{\text{obs}}$	$[\text{Li}]_{\text{obs}}$	$[\text{Li}]_{\text{BB}}$
HD19445	-2.1	7 ± 2	0.4 ± 0.2	2.07 ± 0.07	2.00 ± 0.09
HD140283	-2.6	17 ± 1	-0.1 ± 0.2	2.09 ± 0.07	2.04 ± 0.08
HD201891	-1.3	0.9 ± 0.25	1.7 ± 0.4	1.98 ± 0.07	1.70 ± 0.40

rotational models³¹. The 'high' Be abundances are sometimes considered to be a signature for inhomogeneous nucleosynthesis². Until a plateau can be established for ${}^9\text{Be}$, however, there is no signature, standard or not, for any primordial production of ${}^9\text{Be}$. Furthermore, as the inhomogeneous models cannot alter the conclusions of standard nucleosynthesis significantly when isotope abundance constraints for all light elements $A \leq 7$ are used, it seems unlikely that ${}^9\text{Be}$ will be an exception. Preliminary calculations bear this out^{23,32}. In other words, accurate inhomogeneous nucleosynthesis calculations also do not seem to yield Be/H as high as observed in the population II stars.

Next we come to the stellar models of Pinsonneault *et al.*³¹ with rotation and a reported depletion of primordial ${}^7\text{Li}$ by an order of magnitude. These models consider the possible effects of diffusion beyond standard stellar models arising from large rotationally induced mixing. Pinsonneault *et al.* take a limited high range for stellar angular velocities ranging from 1/20 to 1/2 of the critical break-up value, choose high rotationally induced mixing rates, and find with these assumptions that a primordial value $[\text{Li}] = 3.1$ can be depleted down to a plateau with relatively small dispersion. With such a high value for the primordial abundance, the GCR-produced ${}^7\text{Li}$ would be insignificant. (In the absence of depletion, GCR spallation requires an effective exposure time of ~ 10 Gyr, so although Be suffers much less depletion than Li (ref. 33), the timescale for producing Be by GCR spallation would have to be correspondingly increased, perhaps by an untenable factor as large as 3.) But this model³¹ predicts a dispersion of 0.3 for stellar ages as large as 20 Gyr. For a smaller age of 13 Gyr, the models predict a dispersion of 0.5. Recall that the data show a dispersion, of only 0.12 and this can be accounted for by the observational uncertainties. The chance of this occurring is small for the 20 Gyr model and extremely small for the 13-Gyr model. Clearly, a larger range of angular velocities will produce even more dispersion. An additional failure for these depletion models is that they distinctly predict that the dispersion should be largest at high temperatures; the data do not support this and may contradict it.

In this context, the observation²⁴ of ${}^6\text{Li}$ in the hot halo dwarf HD84937 is clearly important. The rotation models would not allow for the survival of any observable ${}^6\text{Li}$ in this star. Because of the fragility of ${}^6\text{Li}$ (ref. 26), this observation implies that the ${}^7\text{Li}$ in this star is essentially undepleted.

Finally, we note that the current population I stellar abundances of $[\text{Li}] \approx 3$ relative to the primordial abundances of $[\text{Li}] \sim 2$ are easily understood to arise from the addition of ${}^7\text{Li}$ from asymptotic giant branch (AGB) stars (as well as $\sim 10\%$ addition of GCR-spallation produced ${}^7\text{Li}$ that accompanies the production of the observed population I ${}^6\text{Li}$). Considerable enrichments of Li have been observed³⁴ in certain AGB stars. It has been shown³⁵ that reasonable estimates of the frequency of such stars and their subsequent mass-loss would naturally result in Li enhancements in the Galaxy that agree with the observed population I abundance. The production of the AGB Li is presumably via the Cameron-Fowler³⁶ process ${}^3\text{He}(\alpha, \gamma){}^7\text{Be}(\text{beta-decay}){}^7\text{Li}$ in the outer convective zone. Thus a high initial Li is not only unnecessary but could even cause excesses, because the observed AGB Li would have to be added; little of this could be destroyed without also destroying the ${}^6\text{Li}$, for which the only known production mechanism is GCR spallation. The population II Li plateau seems to give us a good estimate of the primordial abundance and fits the simplest model fairly well. $\square$

Received 18 May; accepted 20 October 1992.

1. Rebolo, R., Molaro, P., Abia, C. & Beckman, J. E. *Astr. Astrophys.* **193**, 193–201 (1988).
2. Gilmore, G., Edvardsson, B. & Nissen, P. E. *Astrrophys. J.* **378**, 17 (1992).
3. Ryan, S. G., Norris, J. E., Bessell, M. S. & Deliyannis, C. P. *Astrrophys. J.* (in the press).
4. Gilmore, G., Gustafsson, B., Edvardsson, B. & Nissen, P. E. *Nature* (submitted).
5. Duncan, D. K., Lambert, D. L. & Lemke, M. *Astrrophys. J.* (submitted).
6. Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Kang, H.-S. *Astrrophys. J.* **376**, 51–69 (1991).
7. Spite, F. & Spite, M. *Astr. Astrophys.* **115**, 357–366 (1982).
8. Spite, M., Maillard, J. P. & Spite, F. *Astr. Astrophys.* **141**, 56–60 (1984).
9. Spite, F. & Spite, M. *Astr. Astrophys.* **163**, 140–144 (1986).
10. Hobbs, L. M. & Duncan, D. K. *Astrrophys. J.* **317**, 796–809 (1987).
11. Rebolo, R., Molaro, P. & Beckman, J. E. *Astr. Astrophys.* **192**, 192–205 (1988).
12. Spite, M., Spite, F., Peterson, R. C. & Chaffee, F. H. Jr *Astr. Astrophys.* **172**, L9–10 (1987).
13. Rebolo, R., Beckman, J. & Molaro, P. *Astr. Astrophys.* **172**, L17–19 (1987).
14. Hobbs, L. M. & Pilachowski, C. *Astrrophys. J.* **326**, L23–26 (1988).
15. Hobbs, L. M. & Thorburn, J. A. *Astrrophys. J.* **375**, 116–120 (1991).
16. Reeves, H., Fowler, W. A. & Hoyle, F. *Nature* **226**, 727 (1970).
17. Meneguzzi, M., Audouze, J. & Reeves, H. *Astr. Astrophys.* **15**, 337 (1971).
18. Mitler, H. E. *Astrrophys. Space Sci.* **17**, 186 (1974).
19. Reeves, H. A. *Rev. Astr. Astrophys.* **12**, 437 (1974).
20. Walker, T. P., Mathews, G. J. & Viola, V. E. *Astrrophys. J.* **299**, 745.
21. Steigman, G. & Walker, T. P. *Astrrophys. J.* **385**, L13 (1992).
22. Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Fields, B. *Astrrophys. J.* (submitted).
23. Thomas, D., Schramm, D. N., Olive, K. A. & Fields, B. *Astrrophys. J.* (submitted).
24. Smith, V. V., Nissen, P. E. & Lambert, D. *Astrrophys. J.* (submitted).
25. Deliyannis, C. P., Demarque, P. & Kewaler, S. D. *Astrrophys. J. (Suppl.)* **73**, 21–65 (1990).
26. Brown, L. & Schramm, D. N. *Astrrophys. J.* **329**, L103 (1988).
27. Dearborn, D., Schramm, D. N. & Hobbs, L. *Astrrophys. J.* (in the press).
28. Applegate, J. H., Hogan, C. & Scherrer, R. J. *Phys. Rev. D* **35**, 1151–1160 (1987).
29. Alcock, C., Fuller, G. M. & Mathews, C. J. *Astrrophys. J.* **320**, 439–447 (1987).
30. Kurki-Suonio, H., Matzner, R. A., Schramm, D. N. & Olive, K. A. *Astrrophys. J.* **353**, 406–410 (1990).
31. Pinsonneault, M. H., Deliyannis, C. P. & Demarque, P. *Astrrophys. J. (Suppl.)* **78**, 179–203 (1992).
32. Terasawa, N. & Sato, K. *Astrrophys. J.* **367**, L47 (1990).
33. Deliyannis, C. P. & Pinsonneault, M. H. *Astrrophys. J.* **365**, L67–71 (1990).
34. Smith, V. V. & Lambert, D. *Astrrophys. J.* **345**, L75 (1989); **361**, L69 (1990).
35. Brown, L. *Astrrophys. J.* **389**, 251–268 (1992).
36. Cameron, A. & Fowler, W. *Astrrophys. J.* **167**, 111 (1971).

ACKNOWLEDGEMENTS. This work was supported in part by the DOE at the universities of Minnesota and Chicago, and by NASA and NSF at the University of Chicago. K.A.O. also acknowledges the support of a Presidential Young Investigator Award.

Counter-rotating gaseous disks in the “Evil Eye” galaxy NGC4826

Robert Braun*, René A. M. Walterbos†
& Robert C. Kennicutt Jr‡

* Netherlands Foundation for Research in Astronomy, Postbus 2,
7990 AA Dwingeloo, The Netherlands

† Department of Astronomy, New Mexico State University,
Box 30001/Department 4500, Las Cruces, New Mexico 88003, USA

‡ Steward Observatory, University of Arizona, Tucson, Arizona, USA

SEVERAL elliptical and spheroidal galaxies have been shown, in recent years, to possess kinematically distinct subsystems. These may be in the form of a central stellar component whose rotation is not aligned with the rest of the galaxy^{1,2}, or of a gaseous disk, often at large radius, which is kinematically distinct from the main stellar component^{3,4}. A more unusual case is that of a counter-rotating gaseous component (with some associated stellar absorption) in the inner region of the flattened elliptical NGC4550⁵. All these cases are presumed to be signatures of violently interacting systems, which are the result of galaxy mergers or captures, as in NGC7252^{6,7}. Here we report the discovery of two counter-rotating gaseous disks in the otherwise normal early-type spiral (Sab(s)II; ref. 8) NGC4826. This is the most disk-like galaxy in which any kinematic substructure has yet been found, and our discovery raises the possibility, already suggested by Schweizer⁷, that even spiral galaxies may have undergone a significant degree of structural evolution due to mergers.

NGC4826 (Messier 64) is both nearby (distance $D = 3.8$ Mpc after correcting for Virgo-centric infall and with $H_0 = 100$ km s⁻¹ Mpc⁻¹) and relatively isolated. The galaxy lies at the outskirts of the loose group (CVn I, ref. 9) which includes NGC4736 (M94) and about 15 other possible members. Most of the group members are spirals and irregulars which are spread over an area of 1.9×0.9 Mpc and a velocity range of 300 km s⁻¹. No

nearby companions (within a few degrees) of NGC4826 have yet been identified. A distinguishing feature of the galaxy is the prominent dust-lane pattern on the northeastern side which has led to nicknames such as the “Black Eye” or “Evil Eye” galaxy.

We observed neutral hydrogen emission from NGC4826 with the Very Large Array (VLA)¹⁰ between March 1989 and November 1990 in the B, C and D configurations (with effective integration times of 7, 0.5 and 0.5 hours) to image a region 0.5 degree in diameter at 6 arcsec resolution. In addition, a small hexagonal mosaic in the D configuration (with 0.5 hours effective integration on each of seven positions) was used to image a 1-degree diameter field at 65 arcsec resolution. These observations were part of a program to study the properties of 11 nearby galaxies with high spatial resolution (better than ~ 100 pc) and velocity resolution (6.2 km s⁻¹). Standard calibration and imaging techniques were used to produce a series of narrow-band images separated by 5.15 km s⁻¹ over a velocity range of 660 km s⁻¹ centred on the nominal (heliocentric) systemic velocity of NGC4826, 415 km s⁻¹.

The dominant velocity component of neutral hydrogen emission was determined at both 65 and 15 arcsec resolution by forming an image of the velocity corresponding to the brightest measured emission along each line-of-sight. The resulting velocity fields were ‘blanked’ (declared undefined) outside the connected regions where significant emission was detected. The derived velocity field is shown in Fig. 1. An extended disk (of 22 arcmin diameter) is detected in the low-resolution database

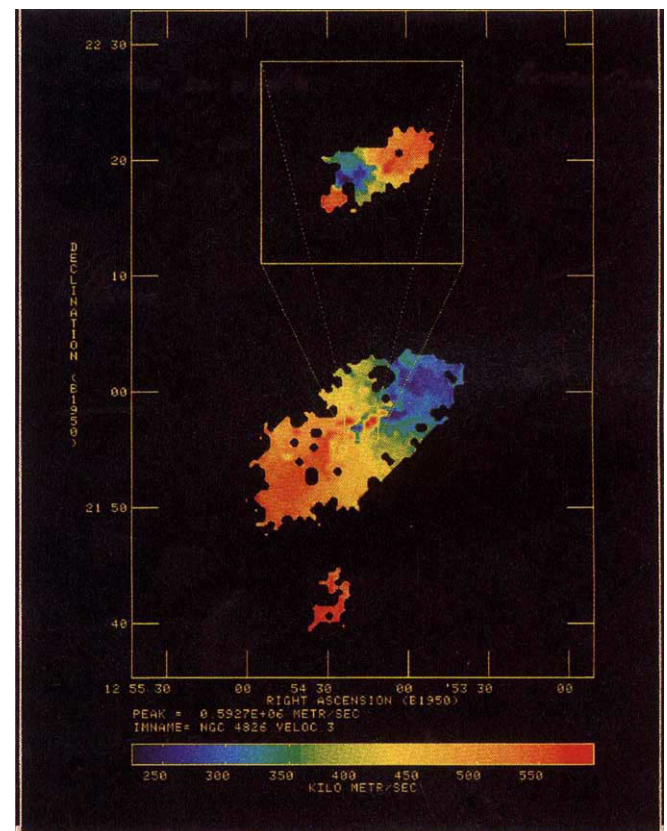


FIG. 1 Line-of-sight velocity of neutral hydrogen emission in the nearby (type Sab(s)II) spiral galaxy, NGC4826. Note the ‘spider pattern’ characteristic of differential rotation, with the highest receding velocities observed along a position angle of 125° (east of north) in the outer disk. The sense of rotation is observed to reverse within the central region of the galaxy, as seen in the high-resolution inset. These counter-rotating gaseous disks are probably the result of a major merger event during the last billion years. Note also the isolated feature 10 arcmin south of the galaxy, which may well represent a remnant of the galactic collision which is still settling into the disk.

consistent with that detected previously¹¹ at a comparable resolution. The more extended spatial coverage of the hexagonal mosaic has revealed at least one additional component of emission situated ~ 10 arcmin south of the extended disk (at velocity 550 km s^{-1}). The relevance of this feature will be discussed below. Fitting a tilted ring model (with the ROTCUR program in the GIPSY software package) to the observed (low-resolution) velocity-field between 100 and 650 arcsec (1.8 and 14 kpc) radius yields the kinematic parameters shown in Fig. 2 (after solving for the mean kinematic centre and systemic velocity). A distortion observed between 100 and 200 arcsec radius is followed by a well-modelled disk rotating at velocity 150 km s^{-1} at an inclination near 65° with a receding line-of-nodes at position angle 125° .

In the inner galaxy, a reversal of the sense of rotation is observed in the central region of 2 arcmin (corresponding to 2 kpc) diameter. Although apparent in the low-resolution data, this central region is best seen at the higher resolution (15 arcsec) of the inset to Fig. 1. Fitting a tilted ring model to the (high-resolution) velocity field between 12 and 60 arcsec yields the kinematic parameters illustrated in Fig. 2 (after solving for the mean kinematic centre and systemic velocity). The rotation velocity is observed to decrease from about 180 to 150 km s^{-1} at an inclination which varies smoothly for about 50 to 70° with a receding line-of-nodes at position angle -70° , opposite to that of the outer galaxy. The magnitude and sense of rotation of this system is fully consistent with that determined through high-velocity-resolution optical spectroscopy of the H II regions in the H α and [N II] emission lines¹². It also agrees with absorption line velocities of a stellar component observed between 3 and 30 arcsec radius (ref. 13). The mass of neutral hydrogen we detect in the inner disk is only 8×10^6 solar masses ($M_\odot$) whereas the total H I mass of the galaxy is $1.4 \times 10^8 M_\odot$ (ref. 11 and references therein). The gas mass in the dusty inner disk is apparently dominated by molecular gas. Integrated line strengths¹⁴ of ^{12}CO ($1 \rightarrow 0$) and ^{13}CO ($1 \rightarrow 0$) are 26.4 ± 0.8 Kelvin km s^{-1} and 5.02 ± 0.24 K km s^{-1} within a 55 arcsec beam that encloses virtually the entire inner disk. Conversion to the associated molecular hydrogen mass assuming the nominal¹⁵ galactic proportionality of column density to ^{12}CO ($1 \rightarrow 0$) flux ($2.3 \times 10^{20} \text{ cm}^{-2} (\text{K km s}^{-1})^{-1}$) yields $M(\text{H}_2) = 1.3 \times 10^8 M_\odot$. Apparently about half of the gas mass in NGC4826 is concentrated within the central disk of 2 kpc diameter.

An immediate question which arises is: what is the actual geometry of these two systems? Although the inclination (with respect to face-on) of each system is determined from the velocity field (if circular rotation of the gas is assumed), the line-of-sight velocity does not, in itself, allow identification of the near and far sides of each disk. On the basis of the kinematic data alone, the inner and outer gaseous disks may be either coplanar to within $\sim 5^\circ$ or misaligned by $\sim 45^\circ$. Some clues to the actual geometry are the following: the prominent dust-lanes in the northeast segment of the inner disk suggest that this is the near side of that system, whereas if the faint optical spiral arms seen at large radii are trailing and share the kinematics of the neutral hydrogen (this needs to be checked with optical spectroscopy), then the higher receding velocity observed at position angle 125° implies that the southwest segment of the outer disk is the near side.

It seems possible, then, that the two gaseous disks we observe are misaligned by $\sim 45^\circ$, so that the two orbital planes may only intersect along the two lines near position angles 120 and 300° rather than continuously. The substantial distortions observed in the outer velocity field at radii less than 150 arcsec may be indicative of the dissipative effects which must accompany any interaction between the two systems. Indeed, whenever similar radii in the two orbital planes become populated, the resulting collisions with relative velocity of 300 km s^{-1} would both heat such gas in the strong resulting shock and strip it of its angular momentum, permitting infall toward the nucleus. The

nucleus of NGC4826 has been classified as a 'LINER' (low ionization nuclear emission-line region) source characterized by relatively strong [N II] emission at $6,584 \text{ \AA}$ and [S II] at $6,717$ and $6,731 \text{ \AA}$ relative to [O III] at $5,007 \text{ \AA}$. This type of nuclear emission is observed in $\sim 80\%$ of early-type spirals and is most often interpreted as evidence for photoionization due to a mildly active nucleus¹⁶, presumably a smaller version of the nuclear sources that power Seyfert galaxies and quasars. The nuclear source in NGC4826 is faint, however, contributing only 8% of the total H α + [N II] flux.

We can estimate the current rate of star formation within the inner disk of NGC4826 from the integrated H α + [N II] flux obtained with large aperture photometry¹⁷, $f(\text{H}\alpha + [\text{N II}]) = 4.8 \times 10^{12} \text{ erg cm}^{-2} \text{ s}^{-1}$. After correction for an assumed 30% contribution from [N II], an 8% nuclear component and a mean extinction of 1.5 mag (estimated from recent spectroscopy we

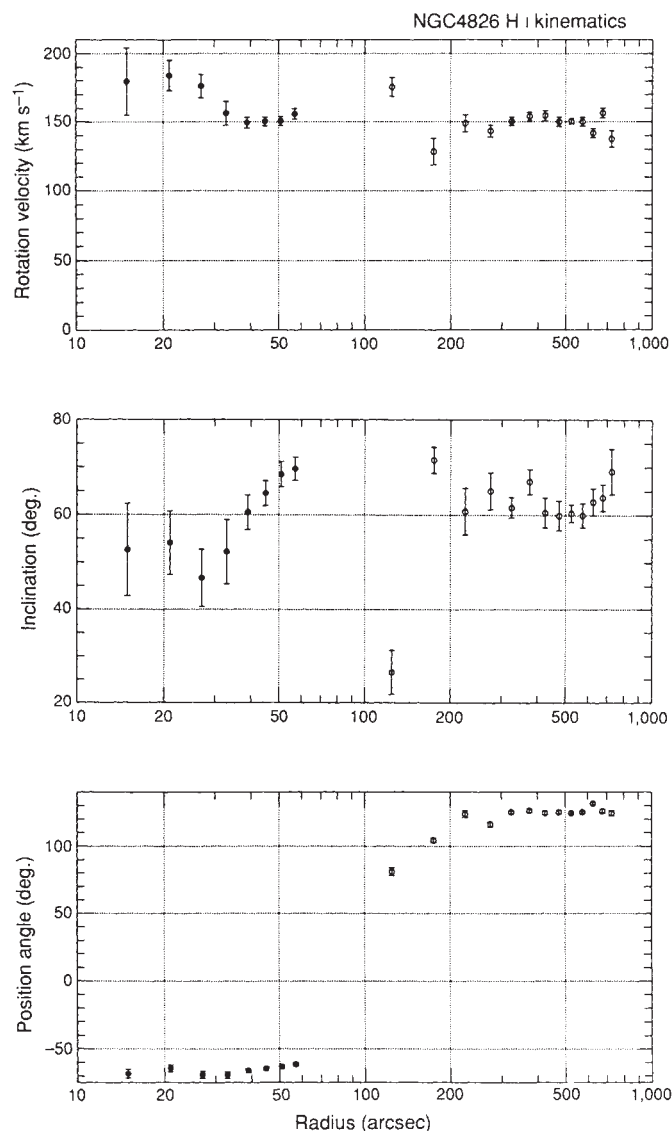


FIG. 2 Kinematic parameters of the counter-rotating neutral hydrogen disks. Rotation velocity, inclination and position angle as a function of radius for the outer disk system ($\circ$) and the inner disk system ($\bullet$), as determined from fitting a tilted ring model to the radial velocities shown in Fig. 1. Note the similar kinematic inclination and rotation velocity but diametrically opposed position angle of the receding line-of-nodes. Also note the severe distortion between 100 and 200 arcsec radius where the two systems may be interacting.

have obtained), we obtain a luminosity $L(H\alpha) = 2.1 \times 10^{40}$ erg s⁻¹. This corresponds to a total star formation rate¹⁸ for the inner disk of $0.19 M_{\odot} \text{ yr}^{-1}$. The associated timescale for depletion of the gas (assuming 25% recycling) is only 0.9 Gyr, making it comparable to that inferred in starburst systems¹⁹.

It is difficult to imagine an intrinsic origin for the counter-rotating gaseous disks that we observe within NGC4826. In view of the dissipative forces which these systems must experience, it seems unlikely that this can be a long-lived phenomenon. An extrinsic origin seems more plausible. Numerical simulations of galactic mergers have begun to incorporate both stellar and gas dynamics^{20,21}. In particular the simulation described in ref. 21, in which two disk galaxies (having bulge: disk: halo mass ratios of 1:3:16 and 10% disk mass in gas) with antiparallel spins approach each other in a plane nearly perpendicular to their spin vectors, strongly resembles the gas distribution in NGC4826. Roughly 1 Gyr (depending on the original galaxy masses and scale lengths) after the initial encounter at an impact parameter of ~ 15 kpc, the simulated galaxies have settled into a merged (semi-)equilibrium state consisting of two nested counter-rotating gaseous disks misaligned by $\sim 10^\circ$. The inner disk is a factor of 7 smaller than the outer disk but contains $\sim 60\%$ of the gas mass and is postulated to undergo a burst of star formation. Scraps of diffuse gas continue to settle into the system from larger radii and scale heights. In the case of NGC4826, the counter-rotating inner disk is (currently) a factor of 12 smaller than the outer disk, contains $\sim 50\%$ of the gas mass, is undergoing a modest burst of star formation and is misaligned by either 5 or 45° . A possible example of diffuse gas still settling into the system is the feature detected to the south of the extended disk of NGC4826.

Although the gas distribution is therefore plausibly accounted for by a merger similar to that considered in the simulation of ref. 21, this is less clear for the stellar distribution. In the simulation the semi-equilibrium stellar distribution is described as "photometrically similar to an elliptical galaxy", whereas broad-band photometry²² of NGC4826 reveals a more complex structure consisting of a compact spheroid and an extended exponential disk accounting for fractions 0.10 and 0.90, respectively of the total B luminosity. The substantial stellar disk component in NGC4826 probably indicates the accretion of a less massive companion rather than the merger of roughly equal-mass systems. A more extensive numerical search of the merger parameter space would be valuable in addressing such issues.

Our discovery of compelling evidence for a recent major merger within a spiral galaxy of type Sab raises a number of interesting questions. How common have major interactions been since the beginning of the galaxy formation process? To what extent are mergers primarily responsible for shaping the Hubble sequence of galaxy types; or more specifically, is the bulge: disk ratio of galaxies some measure of their integrated interaction history? How long-lived are the recognizable signatures of merger events? Finding answers to questions such as these will require substantial effort. It is both encouraging and tantalizing that scrutiny of our closest galactic neighbours is providing additional insight. Messier 31 (classified as an Sb) has been found²³ to have a significantly misaligned (by more than 20°) gaseous disk within the inner few kiloparsecs. Even our own Galaxy (probably an Sbc) has curious kinematic properties within the inner 1.5 kpc which have been interpreted²⁴ as evidence for an inner disk tilted by $\sim 30^\circ$ with respect to the outer disk. Detectable kinematic signatures of merger events, within even apparently 'normal' spiral galaxies, may be much more numerous than we now realize. $\square$

4. Bertola, F., Bettoni, D., Buson, L. M. & Zeilinger, W. W. in *Dynamics and Interactions of Galaxies* (ed. Wielen, R.) 249-252 (Springer, Berlin, 1990).
5. Rubin, V. C., Graham, J. A. & Kenney, J. D. *Astrophys. J.* **394**, L9-L12 (1992).
6. Schweizer, F. *Astrophys. J.* **252**, 455-460 (1982).
7. Schweizer, F. in *Physics of Nearby Galaxies: Nature or Nurture?* (eds Thuan, Balkowski & Tran Thanh Van) (in the press).
8. Sandage, A. & Tammann, G. *A Revised Shapley-Ames Catalog of Bright Galaxies* (Carnegie Institution of Washington, 1981).
9. de Vaucouleurs, G. in *Galaxies and the Universe* (eds. Sandage, A., Sandage, M. & Kristian, J.) 557-600 (Univ. of Chicago Press, Chicago, 1975).
10. Thompson, A. R., Clark, B. G., Wade, C. M. & Napier, P. J. *Astrophys. J. Suppl. Ser.* **44**, 151-167 (1980).
11. Van Driel, W. & Buta, R. J. *Astr. Astrophys.* **245**, 7-26 (1991).
12. Rubin, V. C., Burbridge, E. M., Burbridge, G. R. & Prendergast, K. H. *Astrophys. J.* **141**, 885-891 (1965).
13. Walker, M. F. *Publ. astr. Soc. Pac.* **101**, 333-350 (1989).
14. Sage, L. J. & Isbell, D. W. *Astr. Astrophys.* **247**, 320-334 (1991).
15. Bloemen, H. A. *Rev. Astr. Astrophys.* **27**, 469-516 (1989).
16. Osterbrock, D. E. *Astrophysics of Gaseous Nebulae and Active Galactic Nuclei* 350-352 (Univ. Science Books, Mill Valley, California 1989).
17. Kennicutt, R. C. & Kent, S. M. *Astr. J.* **88**, 1094-1107 (1983).
18. Kennicutt, R. C. *Astrophys. J.* **272**, 54-67 (1983).
19. Kennicutt, R. C. in *The Interstellar Medium in Galaxies* (eds Thronson, H. A. & Shull, J. M.) 405-435 (Kluwer, Dordrecht 1989).
20. Barnes, J. E. & Hernquist, L. E. *Astrophys. J.* **370**, L65-L68 (1991).
21. Hernquist, L. E. & Barnes, J. E. *Nature* **354**, 210-212 (1991).
22. Blackman, C. P. *Mon. Not. R. astr. Soc.* **188**, 93-102 (1979).
23. Braun, R. *Astrophys. J.* **372**, 54-66 (1991).
24. Burton, W. B. & Liszt, H. S. *Astrophys. J.* **225**, 815-842 (1978).

ACKNOWLEDGEMENTS. We thank L. Hernquist for comments on the manuscript and the NRAO for the allocation of observing time on the VLA. The NRAO is operated by Associated Universities Inc. under a NSF Cooperative Agreement. R.C.K. and R.A.M.W. acknowledge grant support from the NSF.

Polyhedral and cylindrical structures of tungsten disulphide

R. Tenne, L. Margulis, M. Genut & G. Hodes

Department of Materials and Interfaces, Weizmann Institute, Rehovot 76100, Israel

FOLLOWING the discovery of C_{60} (ref. 1) and the advent of fullerene chemistry, considerable attention has been directed towards the associated cylindrical^{2,3} and polyhedral^{4,5} forms of graphite. To date, however, observations of such closed structures have been limited to the carbon system. Here we report the formation of equivalent stable structures in the layered semiconductor tungsten disulphide. After the heating of thin tungsten films in an atmosphere of hydrogen sulphide, transmission electron microscopy reveals a variety of concentric polyhedral and cylindrical structures (ranging in size from <10 to >100 nm) growing from the amorphous tungsten matrix. The closed nature of the structures is verified by electron diffraction and lattice imaging. As with the carbon system, complete closure of the tungsten disulphide layers requires the presence of structural defects (for example, edge dislocations), or the arrangement of atoms in polyhedra other than a planar hexagonal geometry.

It is generally believed that geometrically closed structures are unique to carbon because the strain involved for other elements or compounds would not allow a stable structure to exist. An exception is the case of some layered minerals, such as asbestos, which may exist in an open, cylindrical form^{6,7}, rather similar to the concentric tubes of graphite^{3,8,9}. Pauling pointed out in 1930 that an asymmetric layered structure in which the unit cell of one layer differs from that of the second layer (asbestos is one example) is likely to bend because of the asymmetry of the strains on either side of the individual layers¹⁰. He also pointed out that such bending should not be expected for symmetrical layered compounds, such as graphite and MoS_2 . On this basis, the cause of the bending that occurs in asbestos is clearly different from that in graphite.

The analogy between the layered structure of graphite and MX_2 compounds (M is W or Mo; X is S or Se), as shown in Fig. 1, suggests that closed polyhedra may also exist in the latter compounds. We have now observed closed polyhedral and cylindrical crystals of tungsten disulphide (WS_2), a layered

Received 4 September; accepted 22 October 1992.

1. Franx, M., Illingworth, G. & Heckman, T. *Astrophys. J.* **344**, 613-636 (1989).
2. Bender, R. in *Dynamics and Interactions of Galaxies* (ed. Wielen, R.) 232-243 (Springer, Berlin, 1990).
3. Schweizer, F., Van Gorkom, J. H. & Seitzer, P. *Astrophys. J.* **338**, 770-788 (1989).

semiconductor compound. Transmission electron microscope pictures show lattice images of two typical polyhedral growth forms, spherical (Fig. 2a) and elongated (Fig. 2b). Such crystallites occasionally occur in WS₂ films obtained by annealing tungsten films on quartz substrates at 1,000 °C in H₂S in a reducing atmosphere. Figure 2c shows the range of shapes which can occur, and also demonstrates that these structures occur fairly frequently within the layers.

It should be emphasized that all the polyhedral structures are closed in three dimensions. This can be shown by tilting the specimens in the electron microscope: their lattice images remain the same. In cases where non-closed lamellae are adjacent to a polyhedron, the interference fringes of these lamellae disappear on tilting the specimen, while those of the polyhedron are preserved.

Figure 3 shows part of a cylinder of WS₂ with external diameter 16 nm and length of ~200 nm (the smallest cylinder that we observed had four layers and an internal diameter of 4 nm). The free end of the tube is closed and the other end appears to grow out of the surrounding material. The tubular forms were observed to grow in the voids of the otherwise dense material, in contrast with the polyhedral forms of Fig. 2, which grow in the bulk of the material.

Further evidence for the three-dimensional curvature of the crystals is given by typical single-crystal electron diffraction patterns, shown in Fig. 4a (for polyhedra) and 4b (for cylinders). The 6-fold symmetry spot pattern of {hk0} reflections (Fig. 4a) is characteristic of the van der Waals (vdW) plane perpendicular to the electron beam, while the {00.2} ring can be ascribed to vdW planes parallel to the beam. A more complicated diffraction pattern is observed for cylindrical crystals (Fig. 4b), although in this case also, a superposition of {hk0} and {00.2} reflections is obtained. This observation, together with lattice imaging, shows that the crystals are closed. A closed structure will always have some region with vdW plane perpendicular to the electron beam and another region with the vdW plane parallel to the electron beam, thus explaining the presence of both orientations in one crystal.^{2,9}

The stability of the closed crystals is confirmed by the fact that the structures remained essentially unchanged for a period of a year.

A central question is why the WS₂ forms such closed structures. From the energetic point of view, the main stimulus for the closure of sheets would be elimination of dangling bonds at the edges, as was pointed out for graphitic sheets⁵. From the geometrical point of view, complete closure requires replacement of hexagons by other polygonal units, or generation of

structural defects (such as the edge dislocations in Fig. 2a and b).

The experimental results furnish some clues for the growth mechanism. Extensive bending of graphite layers has been reported^{8,11}. Heidenreich *et al.*¹¹ noted that severe bending of graphite layers commonly occurs at high temperatures. Recently, curling and closure of graphitic networks has been observed under electron beam irradiation⁵. Such curling was also observed for MoS₂ (ref. 12) and WS₂ (ref. 13) films. Our WS₂ films are prepared at a fairly high temperature (1,000 °C). This suggests that high temperature or thermal stress may initiate bending or faceting of the crystals. Once the crystal closes on itself (involving strong inter-layer covalent bonding), the structure is expected to be stable, as observed. As for the equivalent graphite structures^{3,8}, the WS₂ crystals may be either rounded or, as is more common, polyhedra with sharp corners. The polyhedron angles vary considerably, again as is the case for the graphite structures⁸. It is difficult to correlate these angles with the crystal structure of WS₂ (although angles close to 120° are often observed). The fuzzy image of the corners of carbon-based polyhedra was interpreted as evidence for the shift of atoms from the position dictated by the *sp*² bond of graphite, and hence suggested the presence of polygons other than hexagons in the corners of carbon-based crystals⁸. Similarly, we often observe a marked loss in the lattice image of the outer corners of the polyhedra (see Fig. 2). We believe this to be due either to an

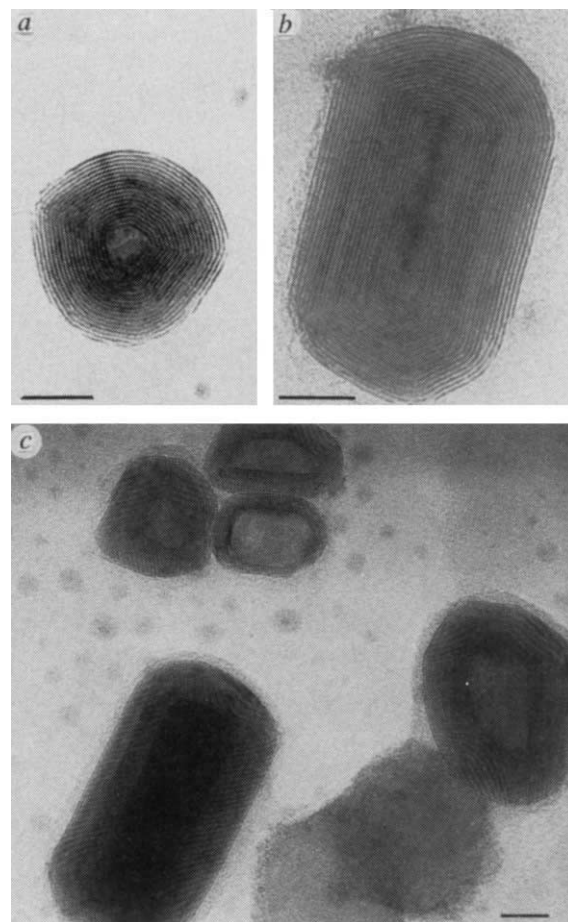


FIG. 2 Electron micrographs of polyhedral crystals of WS₂. a, Spheroidal crystal; b, stir-bar crystal; c, a region containing several polyhedral crystals. The spacing between fringes is 0.615 ± 0.005 nm, which agrees with the distance between two neighbouring layers ($c/2$) of WS₂ (ref. 16). Two plate-like crystals partially overlapping are also seen at the bottom. Scale bar is 10 nm.

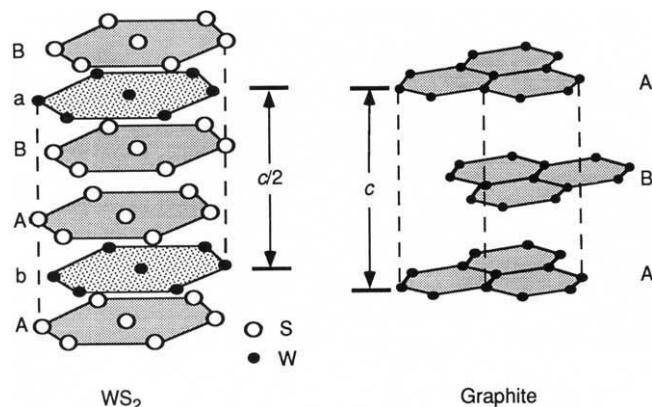


FIG. 1 Layered structure of graphite and WS₂. The graphite is composed of stacking of single layers in which the bonding between the carbon atoms is *sp*². The repeating motif in WS₂ is a sandwich of the three layers S-W-S with covalent tetrahedral W-S bonds and weak van der Waals bonds holding the adjacent sulphur sheets together.

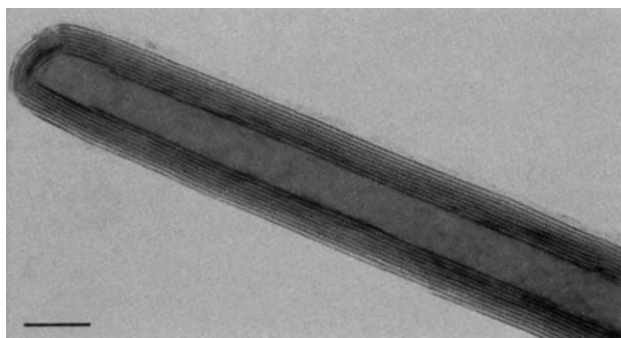


FIG. 3 Electron micrograph of part of a tubular crystal of WS_2 . It is believed to be hollow; the residual contrast inside the tube may result from the outer wall perpendicular to the beam. Scale bar is 10 nm.

irregularity in the crystal structure or to the absence of crystalline material at these edges.

An alternative mechanism for the growth of curved structures may involve the participation of contaminating atoms. For example the structure of graphitic oxide is believed to consist of puckered carbon layers in which the carbon-carbon bonds are roughly tetrahedral¹⁴. This suggests the possibility that the strain of bending graphite (and WS_2) layers is reduced or eliminated by oxide (or some other 'contaminant') formation at the bends⁹. The source of the 'contaminants', such as oxygen species, could be due to diffusion out of the quartz at the high temperatures. Another growth mechanism may involve wrapping a crystalline core with sheets of layered compounds¹⁵. This growth mechanism seems to be irrelevant to the present case, where no crystalline core was observed.

The polyhedral WS_2 structures can join together to give chains or multiple structures, as is clear from the three connected crystals in Fig. 2c. The connection between the two crystals does

not appear to be due to a simple van der Waals attraction, but rather a common crystal plane which undergoes bifurcation in two directions. This could occur by either of two pathways. In the first, two crystallites nucleate close to each other and grow until they merge, with the final sheet common to both of them. The second possibility is that nucleation begins at only one place, and the growing WS_2 sheet splits into two branches, a common occurrence, as we have previously shown (Fig. 7b in ref. 13). The two branches curl round in opposite directions, leading to two connected polyhedra. The bifurcation could occur at any stage in the growth of the initially nucleated crystal. An analogous mechanism has been suggested for the growth of adjacent graphitic spheres from amorphous carbon during high-energy electron irradiation⁵. □

Received 22 July; accepted 11 November 1992.

1. Kroto, H. W., Heath, J. R., O'Brien, C. R., Curl, R. F. & Smalley, R. E. *Nature* **318**, 162-163 (1985).
2. Iijima, S. *Nature* **354**, 56-58 (1991).
3. Iijima, S., Ichihashi, T. & Ando, Y. *Nature* **356**, 776-778 (1992).
4. Kroto, H. W. *Angew. Chem. Int. Edn Engl.* **31**, 111-129 (1992).
5. Ugarte, D. *Nature* **359**, 707-709 (1992).
6. Bates, T. F., Sand, L. B. & Mink, J. F. *Science* **111**, 512-513 (1950).
7. Yada, K. *Acta Cryst.* **23**, 704-707 (1967).
8. Iijima, S. *J. Cryst. Growth* **50**, 675-683 (1980).
9. Bacon, R. *J. appl. Phys.*, **31**, 283-290 (1960).
10. Pauling, L. *Proc. Natn. Acad. Sci. U.S.A.* **16**, 578-582 (1930).
11. Heidenreich, R. D., Hess, W. M. & Ban, L. L. *J. appl. Cryst.* **1**, 1-19 (1968).
12. Moser, J., Liao, H. & Lévy, F. *J. Phys. D. appl. Phys.* **23**, 624-626 (1990).
13. Genut, M., Margulis, L., Hodes, G. & Tenne, R. *Thin Solid Films* **217**, 91-97 (1992).
14. Aragon de la Cruz, F. & Cowly, J. M. *Acta Cryst.* **16**, 531-534 (1963).
15. Bursill, L. A. *Int. J. mod. Phys. B*, **4**, 2197-2216 (1990).
16. *Powder diffraction file, Card 8-237* (ASTM, Philadelphia, Pennsylvania).

Evidence for photochemical control of ozone concentrations in unpolluted marine air

G. P. Ayers*, S. A. Penkett†, R. W. Gillett*, B. Bandy†, I. E. Galbally*, C. P. Meyer*, C. M. Elsworth*, S. T. Bentley* & B. W. Forgan‡

* Division of Atmospheric Research, CSIRO, Private Bag 1, Mordialloc, Victoria 3195, Australia

† School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK

‡ Bureau of Meteorology, Box 1289K, Melbourne 3001, Australia

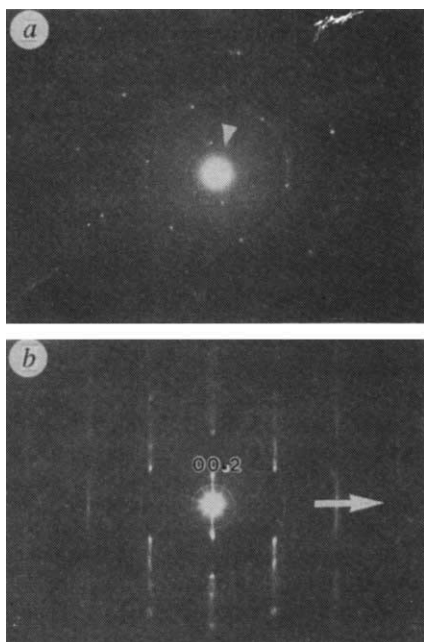


FIG. 4 Typical electron diffraction patterns of (a) a spheroidal crystal; the $\{00.2\}$ ring is arrowed, the other spots belong to $\{hk.0\}$ reflections; (b) a cylindrical crystal. The arrow shows the direction of the cylinder axis. All the reflections are elongated in the direction perpendicular to the cylinder axis, because of the needle-like shape of the cylinders.

OZONE in the troposphere is an important greenhouse gas, and a key participant in the oxidation of other trace species, but the mechanisms for its formation and destruction are not fully understood. In polluted regions of the Northern Hemisphere, seasonal increases in ozone concentration have been observed^{1,2}; such changes could arise from photochemical reactions³⁻⁶, but they could also involve transport from the ozone-rich stratosphere⁷. In remote, unpolluted (low- NO_x) regions, photochemical theory predicts net destruction of ozone⁸. Here we present observations of a large summer minimum in ozone concentration in the unpolluted marine boundary layer of the Southern Hemisphere. Our results show a clear link between ozone loss and hydrogen peroxide production in the region, demonstrating that *in situ* photochemistry, rather than transport, is the major cause of the seasonal ozone cycle in the boundary layer. These findings emphasize the role of photochemical processes in the lower atmosphere, and may suggest only a limited role for transport in other, more polluted regions.

Ozone is both made and destroyed by photochemical reactions in the troposphere, the balance depending on the availability of nitrogen oxides and other gases, particularly hydrocarbons and carbon monoxide. Formation of ozone occurs through

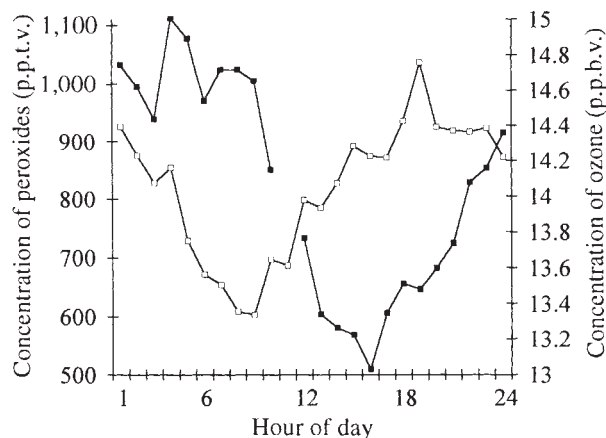
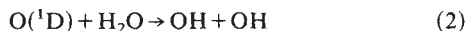
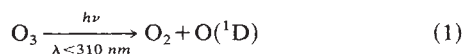


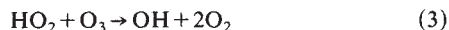
FIG. 1 Average diurnal cycles for peroxide (open squares) and ozone (filled squares) in baseline air at Cape Grim for January 1992.

photolysis of nitrogen dioxide (formed via oxidation of nitric oxide by peroxy radicals), producing oxygen atoms which combine readily with molecular oxygen to yield ozone. This process is well documented in continental areas, where NO_x ($\text{NO} + \text{NO}_2$) concentrations exceed 100 parts per 10^{12} by volume (p.p.t.v.) (ref. 6). In more remote areas, where the NO_x concentration can be extremely low, ozone destruction has been reported⁹ owing to photolysis by near ultraviolet light. This process is not, however, well documented experimentally.

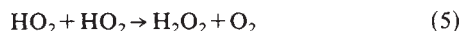
Ozone photolysis in remote, unpolluted (low- NO_x) regions of the troposphere involves a sequence of free-radical reactions:



The hydroxyl radicals produced in reaction (2) can react with carbon monoxide or methane to yield peroxy radicals, for example HO_2 ; these can then react with ozone, as can hydroxyl, leading to further ozone destruction:



Alternatively, two HO_2 radicals can react with each other to produce hydrogen peroxide (H_2O_2):



Organic peroxy radicals, such as CH_3O_2 produced in the course of methane oxidation, also react with HO_2 to form organic peroxides:



We report here the concurrent observation of processes (1)–(6), which we follow by monitoring ozone and peroxide concentrations in surface air. These measurements allow us to assess the extent of photochemically driven peroxy radical chemistry in ozone destruction without the complications of simultaneous ozone-forming NO_x chemistry.

Our measurements were made between February 1991 and March 1992 at the Australian Baseline Air Pollution Station, located at 41°S on the northwestern tip of Tasmania. The station sits on the seaward edge of Cape Grim, at 100 m altitude. This site frequently receives very clean air masses from the Southern Ocean which have had no contact with land for many days. Measurements of peroxides and ozone were made at one-minute intervals except during routine instrument calibration, or failure. Peroxide concentrations were measured using a fluorometric analyser similar to that described by Lazrus *et al.*¹⁰. A TECO

Model 49 ultraviolet photometer was used for the ozone measurements¹¹. The data discussed here were selected according to wind-direction and particle-concentration criteria that have been shown previously¹² to define 'baseline' periods of very clean oceanic air. Details on the station, its scientific programmes and data summaries appear in the Baseline Atmospheric Program annual reports¹³.

Although the peroxide instrument uses two channels to yield data on both total concentrations of peroxides and concentrations with hydrogen peroxide removed enzymatically, we have reservations about the performance of the second channel and so report only total peroxide concentrations, which we suppose to consist primarily of hydrogen peroxide plus some methylhydroperoxide (CH_3OOH). It is generally thought that the latter component should be small (about one-third the concentration¹⁴ of H_2O_2) which, given the lower sensitivity of the instrument¹⁰ for CH_3OOH (by a factor of 0.6), implies that the total peroxide signal should approximate that for hydrogen peroxide alone.

Figure 1 shows the average diurnal variation of ozone and total peroxide in baseline air for January 1992. During sunlit hours, an ozone loss of about 1.6 parts per 10^9 by volume (p.p.b.v.) occurs between mid morning and late afternoon, followed by a replenishment to a similar starting concentration by about midnight. In contrast, the average peroxide concentration increases from about 600 p.p.t.v. to about 900 p.p.t.v. between mid morning and late afternoon, followed by an overnight loss. These diurnal cycles are evident in all months of the summer half-year, but the amplitude of the cycles declines with declining solar intensity towards austral winter (June to August), at which point no clear cycle is discernible above the noise (0.2 p.p.b.v. for ozone and 40 p.p.t.v. for peroxide). These observations leave little doubt that daytime photochemistry simultaneously destroys ozone and produces odd hydrogen radicals (HO and HO_2) in the clean, unperturbed marine boundary layer of the Southern Ocean.

The night-time replenishment of ozone is presumably caused by transport of ozone-rich air from higher altitudes. Although there are no higher-altitude ozone data from Cape Grim to confirm this directly, an eight-year record of ozone profiles presented by Pittcock¹⁵ in the mid-1970s for a coastal site on the Australian continent located 300 km north of Cape Grim shows mean monthly ozone concentrations for the 800-hPa level ($\sim 2 \text{ km}$ altitude), which is consistent with our suggestion. These data and the mean monthly ozone concentrations (1982–1991) from Cape Grim are plotted in Fig. 2. The night-time loss of peroxide must be the result of dry deposition to the ocean surface, which is expected of any highly soluble gas.

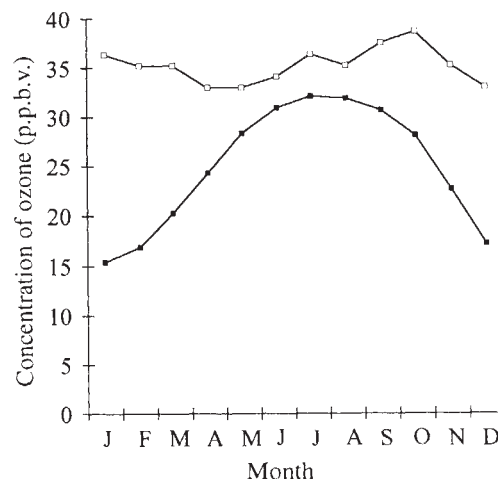


FIG. 2 Monthly mean ozone concentrations at Cape Grim, 1982–91 (filled squares), and at 800 hPa ($\sim 2 \text{ km}$ altitude) over Aspendale, 1965–73 (open squares).

It is important to note that diurnal variations in dry deposition cannot significantly affect the diurnal cycles, because daily cycles in surface temperature, boundary-layer mixing and the mixing height necessary to drive diurnal variations in dry deposition are all very small over the ocean¹⁶. Moreover, the ozone deposition velocity is so low over water ($\sim 0.1 \text{ cm s}^{-1}$)¹⁷ that dry deposition cannot explain the rate of ozone loss apparent in Fig. 1 during morning hours.

Some preliminary quantitative insights can be obtained by writing the rate of change of hydrogen peroxide with respect to time as:

$$\frac{d}{dt}[\text{H}_2\text{O}_2] = k_f[\text{HO}_2]^2 - k_l[\text{H}_2\text{O}_2] \quad (7)$$

Here k_f is the second-order rate coefficient for recombination of HO_2 radicals ($6.7 \times 10^{-12} \text{ cm}^3 \text{ per molecule s}^{-1}$ at 287 K) and k_l is the first-order rate coefficient for physical removal. The latter can be determined from the overnight loss shown in Fig. 1, yielding a first-order rate coefficient of $\sim 1 \times 10^{-5} \text{ s}^{-1}$, equivalent to a deposition velocity of H_2O_2 of about 1 cm s^{-1} . Assuming this applies night and day and is the dominant loss process, it is straightforward to derive an average HO_2 concentration of $3 \times 10^8 \text{ molecules cm}^{-3}$ for the middle six hours of the day. This value may be compared with that predicted by a very simple photochemical box model¹⁸. The modelled processes are photolysis of O_3 to produce $\text{O}(^1\text{D})$, reaction of $\text{O}(^1\text{D})$ with H_2O to yield OH , reaction of OH with CO , CH_4 and O_3 , reaction of H with O_2 to produce HO_2 , reaction of HO_2 with O_3 , NO and itself, and removal of H_2O_2 by photolysis and dry deposition. The model is constrained by observed long-term mean seasonal concentrations of CH_4 , CO , O_3 , H_2O and temperature to predict concentrations of OH , HO_2 and H_2O_2 for each hour of the year. Photolysis rates were computed for clear-sky conditions at the latitude and longitude of Cape Grim, incorporating measured (1987 values) aerosol and ozone column extinctions. The only unconstrained variable is NO concentration, for which we have no data; however, clean air should contain $< 10 \text{ p.p.t.v. NO}$ (ref. 19). As model sensitivity studies showed only minor changes in NO in this range, we assume that the NO concentration remains constant at 3 p.p.t.v.

The predicted HO_2 concentration for the middle six hours of 15 January is $3.6 \times 10^8 \text{ molecules cm}^{-3}$, in good agreement with the 'measured' value (from equation (7)) of $3 \times 10^8 \text{ molecules cm}^{-3}$, especially as the 'measured' value makes no provision for photolytic loss of H_2O_2 and so underestimates HO_2 by about 20%. The daily ozone losses calculated by the model for January are 1.23, 0.2 and 0.13 p.p.b.v. for reactions (2), (3) and (4)

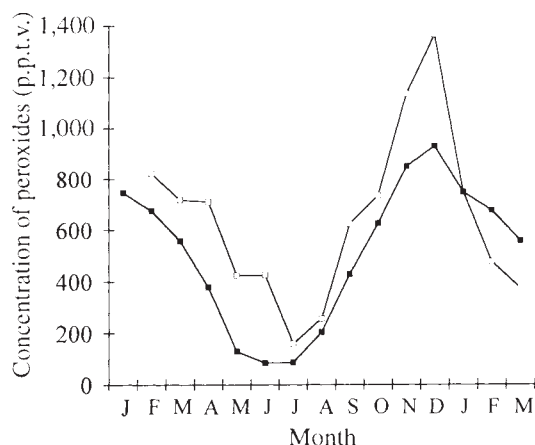


FIG. 3 Comparison of observed (open squares) and model-predicted (filled squares) seasonal cycles of hydrogen peroxide in baseline air at Cape Grim.

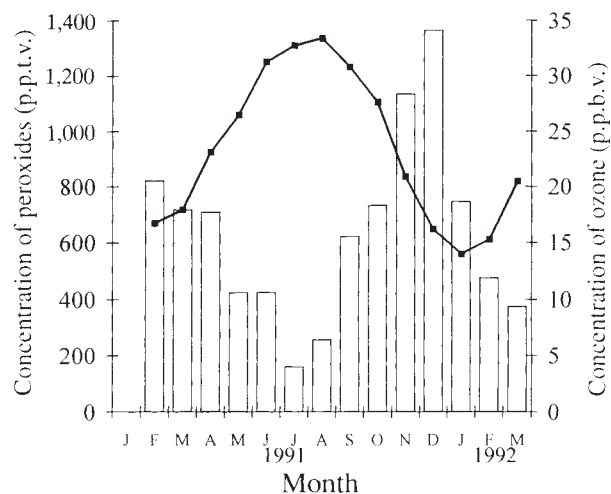


FIG. 4 Comparison of peroxide (open histogram) and ozone (filled squares) seasonal cycles in baseline air at Cape Grim.

respectively giving a total loss of $\sim 1.6 \text{ p.p.b.v.}$ identical to our measured loss. In October the calculated ozone loss is $\sim 0.9 \text{ p.p.b.v.}$, compared with a measured loss of $\sim 1 \text{ p.p.b.v.}$, so despite the simplicity of the model this agreement lends considerable credibility to the basic photochemical scheme.

A comparison of the measured and calculated seasonal cycles of peroxide at Cape Grim is shown in Fig. 3. The agreement is again fairly good, given that the model output does not include organic peroxides and that hydrogen peroxide results from a radical-radical recombination reaction (5) and thus is very sensitive to the predicted HO_2 concentration.

The measured seasonal variations of peroxide and ozone in clean air at Cape Grim during the experiment are contrasted in Fig. 4. The seasonal ozone maximum clearly occurred in winter, when levels of peroxide were minimal, and the minimum average ozone concentration occurred in summer, when maximum concentrations of peroxide were present. These data, when considered in conjunction with the instantaneous loss of ozone and production of peroxide (Fig. 1) show clearly that photochemical processes modulated by the solar cycle control the annual ozone budget of the clean marine boundary layer.

The concentration of ozone in surface air at Cape Grim can now be explained in terms of the competition between vertical mixing in the lower few kilometres of the atmosphere, near-surface destruction by photochemical reactions, and continuous, slow, dry deposition. Assuming that there is little seasonal variation in the ozone mixing ratio at the 800-hPa level (Fig. 2), enhanced photochemical destruction in the moist marine boundary layer in summer must be the cause of both the steep summer minimum in near-surface concentration and an increased vertical gradient. The key factor here is probably enhanced summertime OH formation/ozone loss in the boundary layer (via reaction (2)) caused by the high summertime water-vapour content in the marine boundary layer. This explanation has been advanced previously to explain very similar seasonal cycles at Cape Grim in the vertical gradient of methane, which is destroyed by OH and which, like ozone, shows an enhanced drawdown in boundary-layer concentration in summer²⁰.

The Cape Grim ozone record is very different from those at clean-air sites in the Northern Hemisphere—for example, elevated sites such as Mauna Loa, and Izania in the Canary Islands²¹, and surface sites such as Mace Head on the west coast of Ireland²²—where a spring maximum is a dominant feature. The lack of such a maximum at Cape Grim leaves open the possibility that they may be caused by phenomena other than, or in addition to, increased transport from the stratosphere in

springtime⁷, such as enhanced photochemical production of ozone at that time¹.

Finally, we should comment on the relative abundance of ozone and hydrogen peroxide at Cape Grim. In summer the ozone concentration is ~15 p.p.b.v. whereas the peroxide concentration is ~1 p.p.b.v.. Taking into account their respective lifetimes (1–2 weeks and 1 day), however, the two oxidants are seen to be approximately equal in importance as repositories of highly reactive oxygen. □

Received 13 July; accepted 27 October 1992.

1. Perket, S. A. in *The Changing Atmosphere* (eds. Rowland, F. S. & Isaksen, I. S. A. 38–48) (Wiley, New York, 1988).
2. Fehsenfeld, F. C. et al. *J. Atmos. Chem.* **1**, 87–105 (1983).
3. Levy, H. *Science* **173**, 141–143 (1971).
4. Levy, H. *Planet. Space Sci.* **20**, 919–935 (1972).
5. Crutzen, P. *Pure appl. Geophys.* **106–108**, 1385–1399 (1973).
6. Chameides, W. L. & Walker, J. C. G. *J. geophys. Res.* **78**, 8751–8760 (1973).
7. Levy, H. *J. geophys. Res.* **90**, 3753–3772 (1985).
8. Fishman, J. & Crutzen, P. *Nature* **274**, 855–858 (1978).
9. Johnson, J. E. et al. *J. geophys. Res.* **95**, 121847–11856 (1990).
10. Lazrus, A. L. et al. *Analyt. Chem.* **58**, 594–597 (1986).
11. Elsworth, C. M., Galbally, I. E. & Douglas, M. D. in *Atmospheric Ozone* (eds. Zerefos, C. S. & Ghazi, A.) 809–814 (Reidel, Dordrecht, 1984).
12. Ayers, G. P. & Gras, J. L. *J. geophys. Res.* **88**, 10655–10659 (1983).
13. *Baseline 76–89*, Annual Reports (1976–1989) of the Baseline Atmospheric Program, Australia (Department of Arts, Sport, the Environment, Tourism and Territories, Canberra).
14. Madronich, S. & Calvert, J. G. *J. geophys. Res.* **95**, 5697–5715 (1990).
15. Pittcock, A. B. Q. *J. R. met. Soc.* **103**, 575–584 (1977).
16. Garrat, J. R. *The Atmospheric Boundary Layer* (Cambridge Univ. Press, 1992).
17. Galbally, I. E. & Roy, C. R. Q. *J. R. met. Soc.* **106**, 599–620 (1980).
18. Bentley, S. T. & Ayers, G. P. in *Baseline 89*, 30–37 (Department of Arts, Sport, the Environment, Tourism and Territories, Canberra, 1991).
19. Ridley, B. A., Carroll, M. A. & Gregory, G. L. *J. geophys. Res.* **92**, 2025–2047 (1987).
20. Fraser, P. J. & Hyson, P. *J. Atmos. Chem.* **4**, 3–42 (1986).
21. Schmidt, R. et al. in *EUROTRAC A. Rep. 1990 Part 9* (EUROTRAC International Scientific Secretariat, Garmisch-Partenkirchen, 1991).
22. O'Connor, T. & Simmonds, P. in *EUROTRAC A. Rep. 1991 Part 9* (EUROTRAC International Scientific Secretariat, Garmisch-Partenkirchen, 1992).

ACKNOWLEDGEMENTS. This work was carried out as part of the Tripartite Agreement on Collaborative Studies connected with Climate Change. Funds were provided by the Natural Environment Research Council and the Department of the Environment, UK, the Australian Department of Industry, Trade and Commerce, CSIRO and the Australian Bureau of Meteorology. We thank the staff at Cape Grim for overseeing daily operations of the sampling instruments.

Ice-age atmospheric concentration of nitrous oxide from an Antarctic ice core

Markus Leuenberger & Ulrich Siegenthaler

Physics Institute, University of Bern, Sidlerstrasse 5, 3012 Bern, Switzerland

INCREASING anthropogenic emissions of greenhouse gases are expected to influence the Earth's climate, but the mechanisms for this are not yet fully understood. One way to determine the effect of such gases on climate is to study their atmospheric concentrations during periods of past climate change, such as glacial to interglacial transitions. Previous studies on polar ice cores showed that the concentrations of the greenhouse gases CO₂ and CH₄ were significantly reduced during the last glacial period relative to Holocene values^{1–5}. But no comparable studies have been reported for nitrous oxide (N₂O), which is the next most important greenhouse gas and also affects stratospheric ozone^{6,7} and, potentially, the oxidative capacity of the troposphere⁸. Here we report results from Antarctic ice cores, showing that the atmospheric N₂O concentration was about 30% lower during the Last Glacial Maximum than during the Holocene epoch. Our data also show that present-day N₂O concentrations are unprecedented in the past 45 kyr, and hence provide evidence that recent increases in atmospheric N₂O are of anthropogenic origin.

We have measured the N₂O content in air trapped in the deep ice core from Byrd Station, Antarctica, drilled in 1968 (ref. 9). Ice samples (~500 g) were milled in a dry extraction vacuum system with a milling cutter to open the air bubbles^{10–12}. CO₂ was separated at liquid nitrogen temperature from the extracted air for stable isotope analyses as reported earlier¹³. N₂O, which has a similar vapour pressure at low temperatures, was frozen out together with the CO₂. The CO₂ concentration was measured volumetrically; the small amount of N₂O (~0.1% of the CO₂ amount) is negligible for this measurement. In the combined CO₂ plus N₂O sample, the N₂O/CO₂ ratio was determined by mass spectrometry, and the N₂O/air mixing ratio was then found by multiplying the N₂O/CO₂ ratio by the CO₂/air mixing ratio. The N₂O/CO₂ ratio was determined by measuring the mass ratio 30/28 (NO⁺/CO⁺), taking into consideration the molecular fragmentation patterns of N₂O and CO₂. This N₂O determination method was originally developed to correct the isotopic composition of CO₂ for N₂O (ref. 14).

Extraction and analyses were checked regularly by processing an artificial air standard containing 272 p.p.m.v. CO₂ and (9.7 ± 0.8) p.p.b.v. N₂O together with artificial bubble-free ice. The processed standard air showed enriched N₂O concentrations by

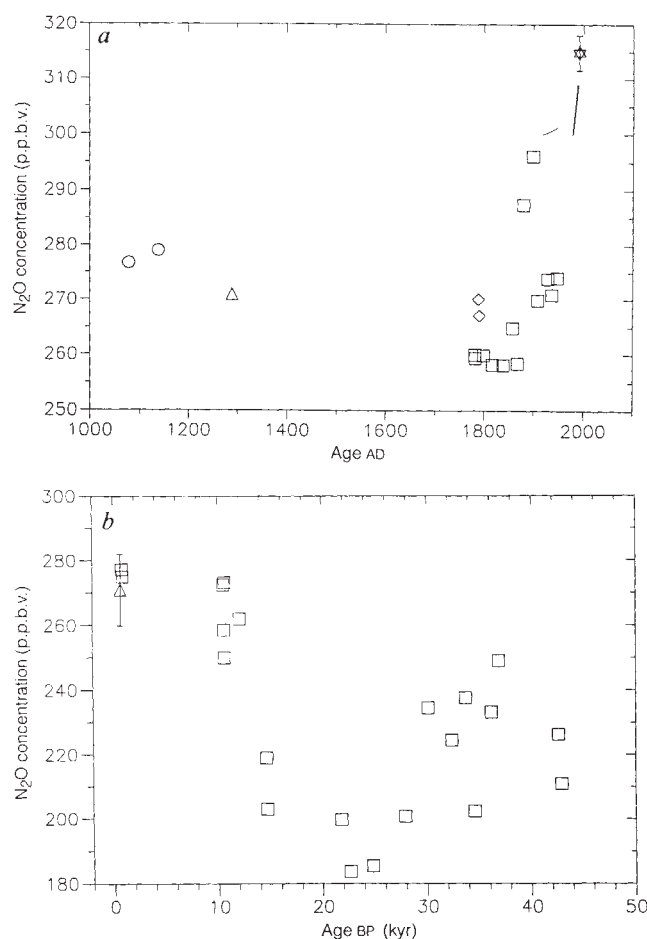


FIG. 1 a, N₂O concentration for the past millennium as measured on the Dye 3 ice core, South Greenland, drilled in 1988 (□), the deep Byrd ice core drilled in 1968 (○) and the shallow Byrd ice core drilled in 1989 (△). Also plotted is the N₂O trend from direct atmospheric measurements for the last 15 years³³ (full line) together with our average value for atmospheric samples from March 1991 (star, see text). Two Dye 3 values with a mean gas age 1788 and 1790 (◇) are considered as unreliable, because the air samples had elevated CO₂ concentration, probably because of melt layers in the ice. b, N₂O concentration from the deep core from Byrd Station, Antarctica, against gas age from ~45,000 yr BP to 800 yr BP (□). The error bar for the youngest sample, from the Byrd ice core drilled in 1989 (△), indicates the typical error.

10 ± 5 p.p.b.v., probably due to friction between metal surfaces in our ice-milling system. Therefore all results were corrected for this excess N_2O . In the same way, we also processed an artificial air standard provided by the Grenoble ice core laboratory, and obtained (261.5 ± 10.3) p.p.b.v., which agrees with the mean measured N_2O concentration of 263 p.p.b.v. obtained by gas-chromatography at Grenoble, by comparing the Grenoble air standard to an air sample provided by CSIRO (Aspendale, Australia), the N_2O value of which was determined relative to an international standard supplied by Oregon Graduate Center. To test the long-term reproducibility of the mass-spectrometric method, we performed weekly tests by measuring four different N_2O/CO_2 standard mixtures, prepared with N_2O/CO_2 ratios ranging from 0.1% to ~6%. This ratio was ~1.0% for the pre-

industrial atmosphere, with N_2O and CO_2 concentrations of ~270 p.p.b.v. and 280 p.p.m.v. The reproducibility of these standard measurements is ~2% for a sample of 10 μ l at standard temperature and pressure, corresponding to our sample size. Adding the error of about ± 5 p.p.m.v. (2–3%) of the volumetrically derived CO_2 concentration¹³ results in an overall uncertainty of the N_2O concentration measurements of 4–5% or 10–12 p.p.b.v. for the ice-core samples.

To test our mass-spectrometric method, we measured the N_2O content of three air samples taken in the undisturbed atmosphere at Jungfraujoch, Switzerland (3,570 m above sea level, m.a.s.l.) in March 1991. The mean value of (314.8 ± 3.2) p.p.b.v. agrees well with atmospheric concentration determined by gas-chromatographic measurements extrapolated to 1991 (311 ± 1 p.p.b.v., ref. 15). We also analysed four aliquots of the Grenoble artificial air standard (mentioned above) without processing them through the ice-milling system, and obtained a mean N_2O concentration of (262.5 ± 3.2) p.p.b.v., in excellent agreement with the expected value.

We have obtained N_2O results covering the past millennium on ice cores from Dye 3, South Greenland, and from Byrd Station (Table 1 and Fig. 1a). The N_2O concentration seems to decrease from ~280 p.p.b.v. in the twelfth century (two samples from the 1968 ice core from Byrd Station) to 260 p.p.b.v. in the eighteenth century (seven values from Dye 3 Station, South Greenland). The only value from an ice core from Byrd Station drilled in 1989 (ref. 16) lies in between (271 p.p.b.v.). Four values in the range of 270–274 p.p.b.v. at the beginning of the twentieth century may point to the industrial increase. We have no explanation for two high outliers with mean gas ages 1879 and 1898. Our results for the pre-industrial N_2O concentration (~260 p.p.b.v.) are lower than those published by other authors^{17,18} who obtained mean values near 285 p.p.b.v. (although a few measurements by Zardini *et al.*¹⁹ yielded ~270 p.p.b.v.). The discrepancy could be due to a systematic offset in our values. The good agreement of our values for present-day atmospheric air with other measurements (Fig. 1a), as well as our different standard tests, argues against such an offset. Therefore, the anthropogenic influence on atmospheric N_2O could be twice as large (increase from 260 to 310 p.p.b.v.) as the 8% increase (from 285 to 310 p.p.b.v.) reported previously.

We measured 25 samples from the 1968 ice core from the Byrd Station⁹ which span the depth interval 146 m to 1,800 m, corresponding to the time period 800 to 43,000 years before present (BP) (C. U. Hammer, personal communication; see Table 1, Fig. 1b). In the early Holocene (~10 kyr BP), we find a N_2O concentration around 265 p.p.b.v., similar to that throughout the Holocene^{17–19}. Lower values are observed in the ice age with a minimum near 190 p.p.b.v. during the Last Glacial Maximum, 20,000 to 25,000 yr BP. A few earlier measurements¹⁹ also indicated lower values during the last glacial-to-interglacial transition. Between 30,000 and 45,000 yr BP, we find intermediate values of 220 to 250 p.p.b.v. The scatter of the early Holocene values is similar to the experimental error, whereas the ice-age values show a larger scatter. We do not know if this variability is due to real fluctuations of the N_2O concentration, as observed for methane⁵, or to poorer experimental reproducibility.

Our results thus indicate that atmospheric N_2O was reduced by ~30% during the Last Glacial Maximum and by ~15% between 30,000 and 45,000 yr BP, compared with the Holocene. These variations must have been caused by changes in the sources or sinks. The main sink for N_2O is in the stratosphere where it is photolysed by ultraviolet radiation (99%) or reacts with excited oxygen atoms $O(^1D)$ (1%)²⁰. N_2O is indirectly responsible for a great part of the destruction of stratospheric ozone^{6,7,20,21}. The lower ice-age N_2O level by itself could therefore have led to a higher stratospheric ozone content than today, which in its turn would have involved reduced ultraviolet intensity in the lower stratosphere and thus slower destruction of N_2O . Increased photolytic destruction of N_2O during the ice

TABLE 1 N_2O concentrations

Byrd core		
Average depth (m)	Mean gas age (yr BP)	N_2O concentration (p.p.b.v.)
133.6	~680	270.9*
146.3	830	277.2
152.7	890	274.9
995.8	10,530	272.4
1,000.7	10,610	250.0
1,001.3	10,620	258.5
1,003.6	10,650	273.1
1,090.6	12,080	261.9
1,203.9	14,610	219.0
1,205.3	14,670	203.0
1,391.4	21,800	199.7
1,410.9	22,640	183.7
1,459.5	24,790	185.4
1,526.8	27,920	200.7
1,571.7	30,090	234.5
1,617.8	32,420	224.5
1,642.8	33,730	237.6
1,658.7	34,570	202.4
1,688.4	36,180	233.2
1,701.1	36,880	249.0
1,798.9	42,590	226.3
1,803.8	42,890	210.9
Dye 3 core		
Average depth (m)	Mean gas age (yr AD)	N_2O concentration (p.p.b.v.)
169.4	1781	260.0
169.2	1782	259.4
166.1	1788	270.1†
165.5	1790	267.1†
160.7	1799	259.9
150.7	1817	258.2
140.7	1839	258.1
130.9	1858	264.8
125.2	1868	258.4
119.9	1879	287.4
110.1	1898	296.1
105.2	1908	269.9
95.1	1928	273.8
90.7	1936	270.9
85.3	1947	274.0

Results for the N_2O concentration for the Byrd ice core and the Dye 3 ice core drilled in 1988. The estimated overall error of the N_2O analyses is 10–12 p.p.b.v. The ages for the Dye 3 core are calculated from H_2O_2 measurements from another Dye 3 ice core drilled in 1986 (A. Sigg, personal communication). We used an air-to-ice age difference of 76 yr. The dating of the Byrd core is subject to uncertainty²⁷; the listed ages are calculated using a depth-age scale of C. U. Hammer (personal communication) for the ice and an air-to-ice age difference of 220 yr.

* The youngest sample from Byrd Station is from an ice core drilled in 1989. Four samples which were contaminated with organic drill fluid, as identified from the mass spectrum, were excluded.

† Two samples from the Dye 3 core are influenced by melt-layer formation, as identified from enriched CO_2 concentration.

age is inconsistent with this argument and therefore seems unlikely. But a change in the two main sources, soils and (to a lesser degree) the oceans^{22,23}, is plausible. A smaller ice-age land biomass (living vegetation and soils) could have led to less N₂O production in soils. A reconstruction of the whole-ocean $\delta^{13}\text{C}$ change, based on deep-sea sediment analyses²⁴, points to a reduction of the land biomass by 500 gigatonnes (Gt) C. From palaeobotanical reconstructions, Adams *et al.*²⁵ estimated that it was reduced by as much as 1,300 Gt C. These estimates correspond to 22–57% of the pre-industrial biomass (including soils) of $\sim 2,300$ Gt C²⁶. Assuming a simple proportionality of biomass size and global N₂O production (admittedly an oversimplification) would lead to a N₂O concentration reduction by the same percentage. Our measurements for the Last Glacial Maximum are $\sim 30\%$ below the Holocene value. Thus, the rough agreement in the size of the two changes is compatible with a causal relationship. Also a change in marine biological processes might have caused or contributed to the lower ice-age N₂O levels.

In Fig. 2, the concentration of N₂O over the past 50,000 years is compared with the records of CO₂ (refs 1, 27) and CH₄ (refs 3, 28), all measured on the Byrd ice core; also shown is the $\delta^{18}\text{O}$ record of this core²⁹. All three trace gases had a lower concentration during glacial time than during the Holocene. Between 30,000 and 50,000 yr BP, the concentrations of N₂O and CH₄ lay between Last Glacial Maximum and Holocene levels, while CO₂ was only slightly higher than in the Last Glacial Maximum. This pattern is consistent with the hypothesis that

the variations of N₂O and CH₄ are both linked to changes in the extent of vegetation and soils (linked to climate and ice coverage), whereas CO₂ varied because of an independent reason (oceanic processes). In particular, the lowest N₂O and CH₄ levels are observed during the Last Glacial Maximum, when the continental ice volume, as recorded by $\delta^{18}\text{O}$ of benthic foraminifera³⁰, had a clear maximum and land biomass probably a minimum.

The change of the N₂O concentration from glacial times to the Holocene must have had an impact on the Earth's radiation balance, N₂O being an infrared-active gas. An evaluation of the direct greenhouse effect of the N₂O change²² yields a global radiative forcing of $\Delta F = 0.36 \text{ W m}^{-2}$ for the N₂O increase from 190 (Last Glacial Maximum) to 270 p.p.b.v. (Holocene). Compared with the direct radiative effects of the other greenhouse gas changes (CO₂: $\Delta F = 2.44 \text{ W m}^{-2}$ for 190 to 280 p.p.m.v.; CH₄: $\Delta F = 0.22 \text{ W m}^{-2}$ for 350 to 650 p.p.b.v.), N₂O contributes 15% as much as CO₂ and is more important than CH₄. For CH₄, indirect chemical feedbacks (tropospheric ozone, stratospheric water vapour) must be added (ref. 5), but the strength of these chemical feedbacks is still in question³¹; Lelieveld and Crutzen³² recently argued that these chemical feedbacks (including the stratospheric water vapour effect which applies for the glacial-interglacial difference) have been overestimated. In that case, N₂O must have been roughly as important as methane in radiative forcing during the last glacial-to-interglacial change. At any rate, N₂O and CH₄ must have contributed at least 30% as much as CO₂ to the glacial-postglacial change in greenhouse forcing. □

Received 23 June; accepted 20 October 1992.

1. Neftel, A., Oeschger, H., Staffelbach, T. & Stauffer, B. *Nature* **331**, 609–611 (1988).
2. Barnola, J. M., Raynaud, D., Korotkevich, Y. S. & Lorius, C. *Nature* **329**, 408–414 (1988).
3. Stauffer, B., Lochbrunner, E., Oeschger, H. & Schwander, J. *Nature* **332**, 812–814 (1988).
4. Raynaud, D., Chappellaz, J., Barnola, J. M., Korotkevich, Y. S. & Lorius, C. *Nature* **333**, 655–657 (1988).
5. Chappellaz, J., Barnola, J. M., Raynaud, D., Korotkevich, Y. S. & Lorius, C. *Nature* **345**, 127–131 (1990).
6. Hahn, J. & Crutzen, P. J. *Phil. Trans. R. Soc. Lond.* **B296**, 521–541 (1982).
7. Crutzen, P. J. *Tellus* **26**, 47–57 (1974).
8. Staffelbach, T., Neftel, A., Stauffer, B. & Jacob, D. *Nature* **349**, 603–605 (1991).
9. Ueda, H. T. & Garfield, D. *Tech. Rep. 231* (U.S. Army Corps of Engineers, Cold Regions Research and Engineering Laboratory, Hanover, New Hampshire, 1969).
10. Moor, E. & Stauffer, B. *J. Glaciol.* **30**, 358 (1984).
11. Friedli, H., Moor, E., Oeschger, H., Siegenthaler, U. & Stauffer, B. *Geophys. Res. Lett.* **11**, 1145–1148 (1984).
12. Friedli, H., Löttscher, H., Oeschger, H., Siegenthaler, U. & Stauffer, B. *Nature* **324**, 237–238 (1986).
13. Leuenberger, M., Siegenthaler, U. & Langway, C. C. *Nature* **357**, 488–490 (1992).
14. Friedli, H. & Siegenthaler, U. *Tellus* **B40**, 129–239 (1988).
15. Aleya, F. N. *et al.* in *Trends 91* (eds Boden, T. A., Sepanski, R. J. & Stoss, F. W.) 352–369 (CDIAC, Oak Ridge National Laboratory, Tennessee, 1991).
16. Langway, C. C. & Osada, K. *Proc. Symp. Tropospheric Chem. of the Antarctic Region*, June 3–6 (Univ. of Colorado, Boulder, 1991).
17. Khalil, M. A. K. & Rasmussen, R. A. *Ann. Glaciol.* **10**, 73–79 (1988).
18. Etheridge, D. M., Pearman, G. I. & de Silva, F. *Ann. Glaciol.* **10**, 28–33 (1988).
19. Zardini, D., Raynaud, D., Scharffe, D. & Seiler, W. *J. Atmos. Chem.* **8**, 189–201 (1989).
20. Seinfeld, J. H. *Atmospheric Chemistry and Physics of Air Pollution* (Wiley, New York, 1986).
21. Wayne, R. P. in *Chemistry of the Atmosphere* (ed. Wayne, R. P.) 171–178 (Oxford Univ. Press, 1991).
22. Intergovernmental Panel on Climate Change. *Climate Change—The IPCC Scientific Assessment* (eds Houghton, J. T. *et al.*) (Cambridge Univ. Press, 1990).
23. Cicerone, R. J. *J. Geophys. Res.* **94**, 18265–18271 (1989).
24. Duplessy, J. C. *et al. Paleoceanography* **3**, 343–360 (1988).
25. Adams, J. M., Faure, H., Faure-Denard, L., McGlade, J. M. & Woodward, F. I. *Nature* **348**, 711–714 (1990).
26. Bolin, B. in *The Greenhouse Effect, Climatic Change, and Ecosystems* (eds Bolin, B. *et al.*) 93–156 (Wiley, Chichester, 1986).
27. Staffelbach, T., Stauffer, B., Sigg, A. & Oeschger, H. *Tellus* **B43**, 91–96 (1991).
28. Lochbrunner, E. thesis, Univ. of Berne (1989).
29. Johnson, S., Dansgaard, W., Clausen, H. B. & Langway, C. C. *Nature* **235**, 429–434 (1972).
30. Labeyrie, L. D., Duplessy, J. C. & Blanc, P. L. *Nature* **327**, 477–482 (1987).
31. Isaksen, I. S. A., Ramaswamy, V., Rodhe, H. & Wigley, T. M. L. in *Climate Change 1992, The Supplementary Report to the IPCC Scientific Assessment*, 47–67 (Cambridge Univ. Press, 1992).
32. Lelieveld, J. & Crutzen, P. J. *Nature* **355**, 339–342 (1992).
33. Elkins, J. W. & Rossen, R. in *Summary Report 1988, Geophysical Monitoring for Climatic Change* (NOAA ERL, Boulder, Colorado, 1988).

ACKNOWLEDGEMENTS. We thank C. C. Langway Jr for the Byrd ice core samples, M. Töngi for assistance with mass spectrometry, A. Neftel and D. Raynaud for discussions, and J. Schwander and H. Oeschger for advice and encouragement. The Byrd ice core samples are part of a University of Bern and SUNY at Buffalo collaboration supported by the U.S. NSF/DPP. We thank our colleagues from Bern who drilled the shallow ice core at Dye 3 in 1988, A. Sigg for providing the H₂O₂ results for dating this core, and D. Raynaud and T. Martin for their artificial N₂O-in-air standard. This work was supported by the Swiss NSF.

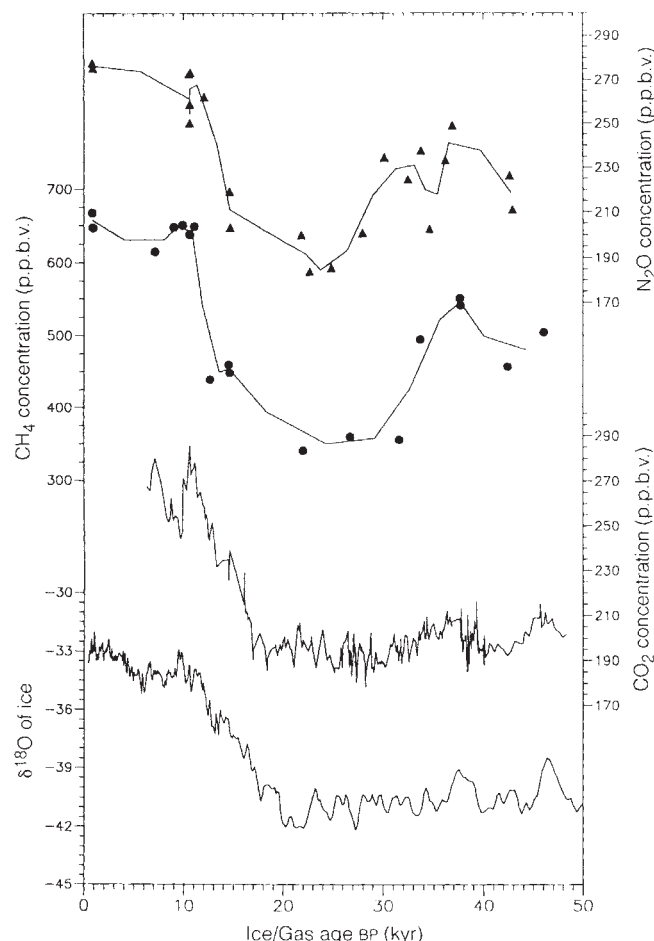


FIG. 2 Concentrations of the three gases N₂O, CH₄ (ref. 3, 28) and CO₂ (refs 1, 27) against gas age, and $\delta^{18}\text{O}$ (ref. 29) against age of the ice, all measured on the Byrd ice core. Solid lines represent two-point running means. $\delta^{18}\text{O} = (^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}} - 1$ where standard is SMOW.

Excitation of true polar wander by subduction

Giorgio Spada^{*†}, Yanick Ricard^{*}
& Roberto Sabadini[†]

^{*} Département de Géologie, de l'Ecole Normale Supérieure,
75231 Paris, France

[†] Istituto di Geofisica, Dipartimento di Fisica, Università di Bologna,
40127 Bologna, Italy

TRUE polar wander—the global motion of the mantle relative to the Earth's rotation axis—is known from the analysis of palaeomagnetic data, hotspot tracks and plate motions to have occurred at velocities of up to $0.5^\circ \text{ Myr}^{-1}$ since the Late Cretaceous period^{1–4}. We address here the longstanding question of how fast episodes of true polar wander (TPW) can be excited⁵, by analysing the impact of the distribution and activity of subduction zones on polar motion. Using nonlinear Liouville equations, which allow us to treat large excursions of the polar axis, we show that unrealistically fast TPW is excited by subduction episodes unless the lower mantle has a viscosity at least 10 times that of the upper mantle. This need for a viscosity increase with depth in the mantle reinforces the conclusions of previous studies on post-glacial rebound and geoid anomalies, theoretical creep laws and some preliminary results on TPW induced by density anomalies embedded in the mantle^{6–10}. The lower viscosity in the upper mantle means that upper-mantle density anomalies are most effective in exciting TPW. Changes in the pattern of subduction through time may be responsible for both episodes of fast TPW and times of quiescence in polar motion.

The 1969 paper of Goldreich and Toomre¹¹ renewed the interest of the geophysical community¹² in the role of polar wandering in Earth's dynamics. Their example of a colony of beetles crawling on the surface of a quasi-rigid Earth suggests that large excursions of the axis of rotation with respect to the entire mantle could be produced by small redistributions of matter. A large amount of TPW can be caused by the cumulative effects of the random walk of tiny density features, mimicking drifting lithospheric plates¹¹.

Goldreich and Toomre made two main approximations, neglecting isostatic compensation and the Earth's equatorial bulge. These two approximations, valid for a quasi-rigid planet, are abandoned here as we use a viscoelastic stratified model. When mantle rheology is considered, isostasy cancels the inertial perturbations associated with surface tectonic features so that

drifting plates cannot excite polar wander. On the contrary, geoid anomalies suggest that mantle density heterogeneities embedded sufficiently far away from chemical interfaces, such as the Earth's surface, the core-mantle boundary (CMB) or the 660-km discontinuity, induce the main perturbation of the inertia tensor^{13–15}.

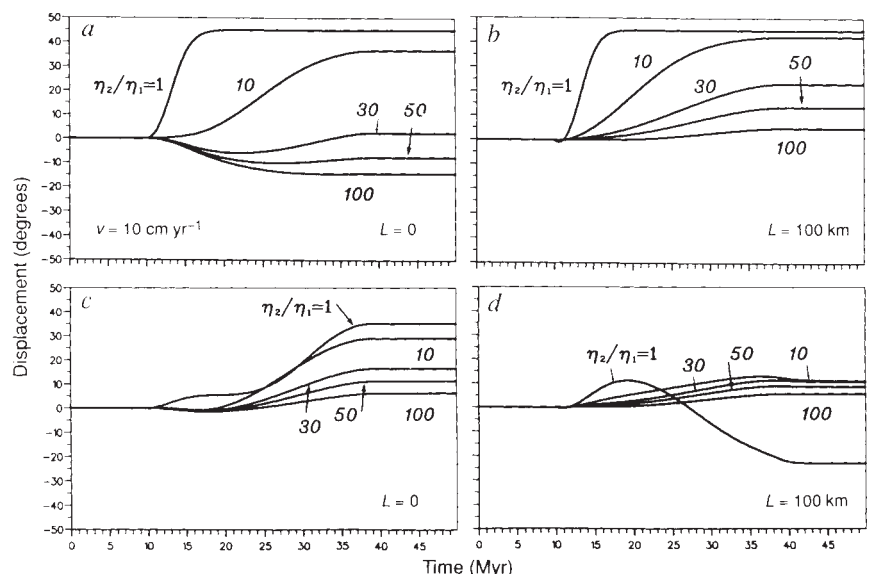
To understand the effect of mantle mass redistribution in exciting TPW, we solve the nonlinear Liouville equations that govern the Earth's rotation in the case of inertia perturbations due to time-dependent subduction processes. The inertia tensor $J_{ij}(t)$ of a rotating body can be organized as follows¹⁶

$$J_{ij}(t) = I\delta_{ij} + \frac{k^T(t)a^5}{3G} [\omega_i(t)\omega_j(t) - \frac{1}{3}\omega^2(t)\delta_{ij}] + [\delta(t) + k^L(t)]C_{ij}(t) \quad (1)$$

where ω_i denotes the components of the angular velocity vector in a geographical frame, G the gravitational constant, a the Earth's radius and I the inertia of the non-rotating planet. A perturbation $C_{ij}(t)$ acts instantaneously through a Dirac delta function $\delta(t)$ and has a delayed response expressed by the convolution with the loading Love number $k^L(t)$ (ref. 17). The viscous readjustment of the equatorial bulge is described by the term containing the tidal Love number $k^T(t)$. For a viscoelastic planet, the two Love numbers k^L and k^T , characterized by a set of modes and their own relaxation times, are obtained by means of classic analytical techniques¹⁸ and account for the response of the Earth to loading and tidal potentials. For a model consisting of an elastic lithosphere, an upper and lower mantle described by a viscoelastic Maxwell rheology, and an inviscid core¹⁹, the time dependence of the Love numbers introduces into the Liouville equations widely separated timescales ranging from the fast Chandler wobble oscillation to the relaxation over several million years of internal chemical boundaries^{14,15,20}. These various characteristic times prohibit any numerical treatment of the Liouville equations unless some approximation is made. As the subduction zones only evolve with a timescale of a few million years, we perform an asymptotic expansion of the two Love numbers for large time. Such an expansion filters the fast modes and allows us to solve the Liouville equation by a Runge-Kutta scheme.

We model the subduction processes by the sinking of point sources of mass $2 \times 10^{19} \text{ kg}$ in the mantle. This corresponds to a slab pull of $5 \times 10^{13} \text{ N m}^{-1}$ along a trench²¹ whose total length is $4 \times 10^3 \text{ km}$. The inaccuracy in the inertia tensor due to the use of point masses instead of extended slabs is negligible. We

FIG. 1 Displacement of the axis of rotation for a mass anomaly sinking at a constant rate of 10 cm yr^{-1} down to the CMB, for varying viscosity ratio η_2/η_1 . The initial latitude of the source, activated at $t=10 \text{ Myr}$, is 45° . a, b, Phase-change transition at 660 km depth; c, d, Chemical boundary with a density jump $\Delta\rho/\rho=9\%$. The lithosphere is absent in a and c, whereas its thickness is fixed at 100 km in b and d.



impose $C_{ii}(t) = 0$ to conserve the average inertia of the Earth²² and its mass.

Figure 1 portrays the angular displacement $m = \arccos(\omega_1/\Omega)$ (in degrees, where Ω denotes the diurnal angular velocity) of the axis of rotation induced by a single subducting slab activated at 10 Myr at a latitude of 45° and sinking at a velocity of 10 cm yr^{-1} down to the CMB. The upper-mantle viscosity η_1 is kept to 10^{21} Pa s , while the lower-mantle viscosity η_2 is varied from η_1 to $100\eta_1$. Panels *a* and *b* correspond to boundary conditions at the upper-lower mantle interface appropriate for a fully adiabatic phase-change transition. A non-adiabatic chemical transition at 660 km is modelled in panels *c* and *d*. In such a stratified mantle the slab does not penetrate the lower mantle. We assume, however, for the sake of simplicity, that thermal coupling through the 660-km interface induces a cold, sinking blob with the mass of the slab. The lithosphere is absent in Fig. 1 *a* and *c* with $L = 0$, whereas its thickness is fixed at 100 km in Fig. 1 *b* and *d*.

We choose the convention that positive TPW values denote a drift toward the mass anomaly. There is a dominance of such motions that indicates that the inertia associated with topography generated by compensation on top of the slab generally overcomes the inertia perturbation due to the slab itself. Surface compensation is inhibited to some extent by the hardening of the lower mantle, and for $\eta_2/\eta_1 = 50$, $L = 0$ (ref. 8), the rotation pole is forced in the opposite direction (Fig. 1*a*). The perturbations in the moment of inertia are associated with the geoid components of degree 2 (ref. 10) and the rotation pole wanders toward a geoid low and away from a geoid high. In an isoviscous mantle, the pole position is highly unstable, as shown by the rapid 45° displacement in Fig. 1*a*. Viscosity increase with depth or chemical stratification reduces the displacement amplitude and increases the time span needed to attain the equilibrium. Linearized Liouville equations, considered previously¹⁰, are correct only for the small excursions of the axis of rotation occurring in the early stages of subduction (Fig. 8 of ref. 10) and cannot be used to model realistic subduction episodes which can in principle cause large amounts of TPW, as shown in this figure.

We can now generalize the results of Goldreich and Toomre¹¹ by computing the pole displacement generated by a random distribution of density anomalies that model episodic subduction processes in a viscously stratified Earth. Such a model of mass redistribution is simplified, but we think that it captures the main physical ingredients of TPW induced by real subductions.

We consider subduction episodes activated every $\Delta t = 2 \text{ Myr}$ (Fig. 2*a–d*) and every $\Delta t = 10 \text{ Myr}$ (Fig. 2*e, f*). Their geographical coordinates are chosen by means of a random number generator. The process of density anomaly generation is interrupted at 500 Myr. The slab velocities in the upper mantle are 10 cm yr^{-1} except for the dotted lines of Fig. 2*e* and *f* where they have been reduced to 5 cm yr^{-1} . The value of 10 cm yr^{-1} , an upper bound for the present-day subduction pattern, is appropriate for the Late Cretaceous, when plate velocities and spreading rates were generally higher^{23,24}. In all these computations, the mantle is adiabatically stratified and the slab velocities are diminished in the lower mantle in accordance with the viscosity increase.

Figure 2*a* and *b* shows, for a mantle with uniform viscosity, the absolute velocity V and the three components of the angular velocity vector ω_i/Ω in the geographical coordinate frame with the North Pole coincident with the rotation pole at $t = 0$. Their values are extremely sensitive to the distribution of the internal density anomalies. The TPW velocity can be as high as $15^\circ \text{ Myr}^{-1}$ during the periods of largest rotational instabilities. This is much larger than the value inferred from palaeomagnetic studies, $0.5^\circ \text{ Myr}^{-1}$, indicated by a horizontal arrow.

For $\eta_2/\eta_1 = 10$ the mantle flow is inhibited and the average pole velocity is lowered to $\sim 0.5^\circ \text{ Myr}^{-1}$ (Fig. 2*c, d*). The spikes

are smoother than in Fig. 2*a*, about 1° Myr^{-1} , because of the enhanced rotational stability. The transient of about 50 Myr (Fig. 2*c, d*) is a consequence of the initial lack of any isotropy in the geographical distribution of subductions; this effect is also visible in Fig. 2*a* and *b*, on a shorter timescale because of the lower viscosity. Another deviation from the results of the isoviscous mantle is the decreased variability in the velocity V and in the ω_i/Ω components, after most of the subducting slabs have crossed the 660-km viscosity discontinuity at 500 Myr.

In Fig. 2*e* and *f* the interval Δt between subduction episodes is increased to 10 Myr, the largest time interval that ensures that at least one subduction is always active in the upper mantle, even with a sinking velocity of 5 cm yr^{-1} . The number of subduction episodes is kept constant so that the process of subduction generation covers the whole time window shown in the figure.

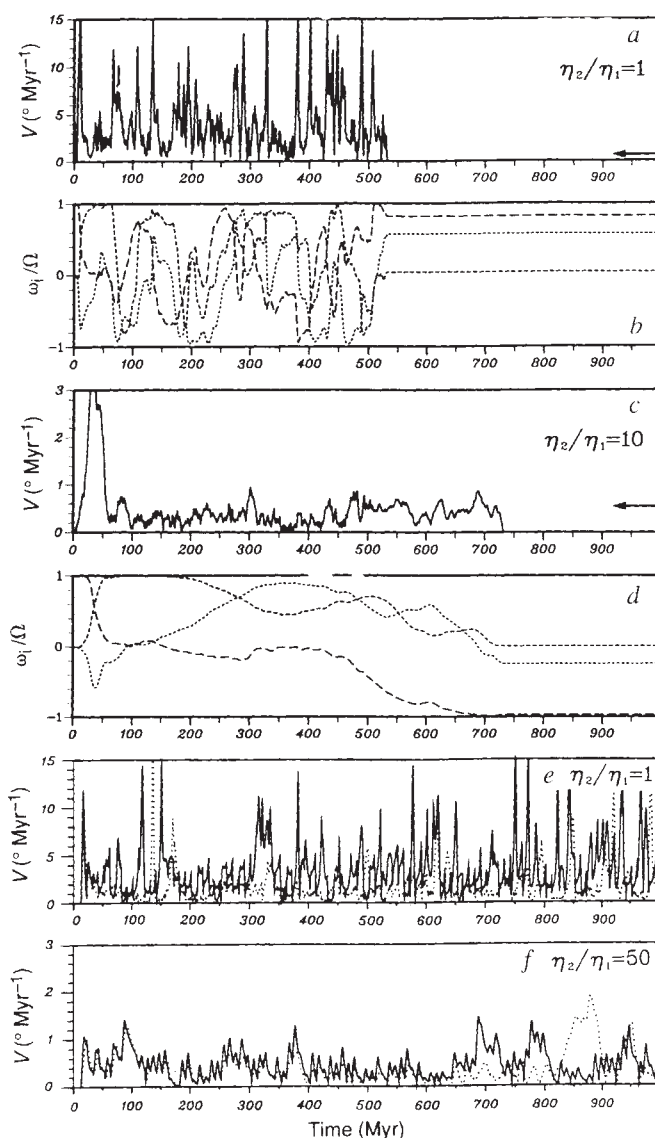


FIG. 2 *a–d*, Absolute value of polar wander velocity V ($^\circ \text{ Myr}^{-1}$) and *b, d*, normalized components of the angular velocity vector ω_i/Ω , for different lower-mantle viscosities and phase-change transition; the dotted curves in *b* and *d* denote the equatorial components of the angular velocity vector while the dashed ones stand for the polar component. The velocity of the blobs in the lower mantle is reduced in accordance with the viscosity increase. In *a* and *d*, density anomalies are activated randomly every 2 Myr, whereas in *e* and *f*, Δt is increased to 10 Myr. $\eta_2/\eta_1 = 1$ in *a, b* and *e*, $\eta_2/\eta_1 = 10$ in *c* and *d*, and $\eta_2/\eta_1 = 50$ in *f*. Dotted curves in *e, f* stand for sinking velocity of 5 cm yr^{-1} . The horizontal arrows denote the TPW velocity inferred from observations^{1–3}.

Comparison with Fig. 2a indicates that the results are essentially insensitive to the activation time Δt of subduction episodes, suggesting that TPW is controlled by the timescale associated with the readjustment of the equatorial bulge. We verified that with $\Delta t = 50$ Myr the subducting slabs do not interact and the case of the single episode of Fig. 1 is retrieved. A decrease in the slab velocity to 5 cm yr^{-1} (dotted) reduces the TPW for the uniform mantle as shown in Fig. 2e. This velocity, however, is still much larger than that observed. A large viscosity increase (Fig. 2f) suppresses this dependence of TPW on the sinking velocity of the slabs.

The 660-km transition is treated as a chemical boundary in Fig. 3. For $\eta_2/\eta_1 = 1$ or 10 the results are similar to the phase-change models of Fig. 2, the peak values for the velocity are generally smaller. The chemical boundary reduces the mantle flow, decreasing the variability of the moment of inertia. This effect is more pronounced when the lower-mantle viscosity is increased (Fig. 2e, f). After all the density anomalies have entered the highly viscous lower mantle, the Earth becomes stable and the random characteristics of the pole path tend to vanish (Fig. 2f). The decoupling of the mantle flow, enhanced

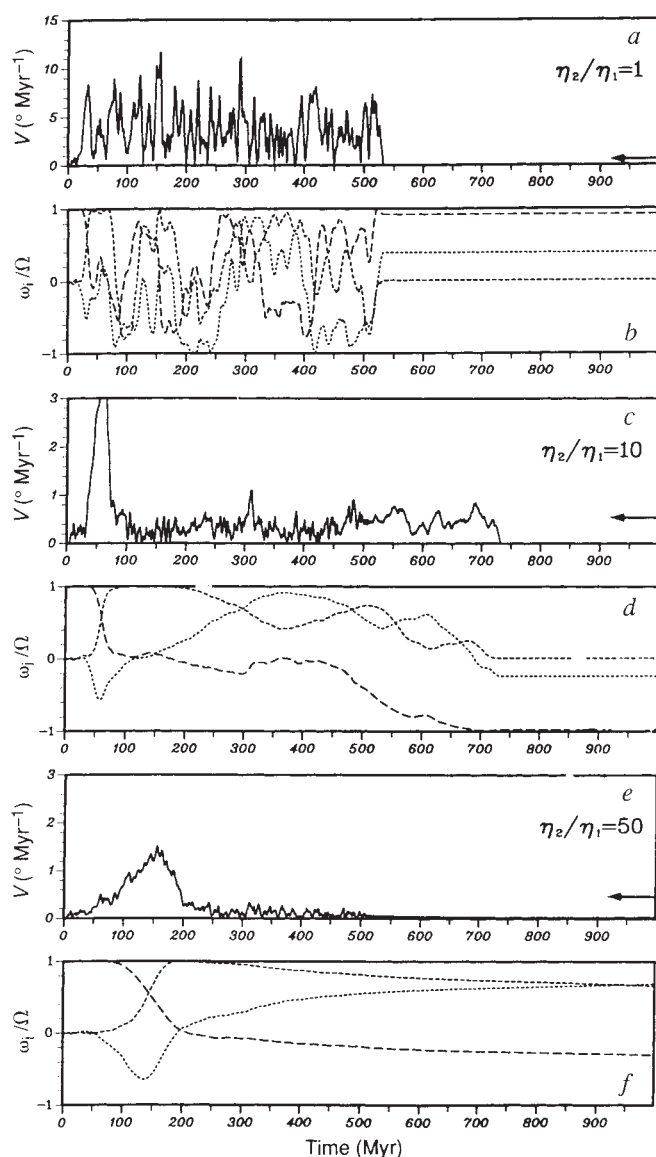


FIG. 3 a-d, Same as in Fig. 2 except for non-adiabatic boundary conditions at 660 depth with $\Delta\rho/\rho = 9\%$. In e and f, the viscosity contrast η_2/η_1 is increased to 50.

by the chemical density jump, acts as a stabilizing factor when the density anomalies are still active in the upper mantle.

These results show that changes in the geographical distribution of subduction zones could induce considerable polar wander. The viscosity profile and the density contrasts at 660 km depth control the TPW. The observations suggest that the lower-mantle viscosity should be at least 10^{22} Pa s . The hardening below 660 km shields the upper mantle and damps the flow forced by lower-mantle density anomalies, suppressing their effects on TPW. Chemical stratification enhances this behaviour. Our findings provide the geodynamical basis for the interpretation of the fast TPW during the early Tertiary, synchronous with major tectonic events that led to a marked increase in the length of collisional plate boundaries and changes in the location of subduction zones^{3,25}. Standstills in TPW are not necessarily related to a reduction in global tectonic activity, but rather to random anomalous density distributions with vanishing total moment of inertia. □

Received 17 March; accepted 1 October 1992.

- Gordon, R. G. & Livermore, R. A. *Geophys. J. R. astr. Soc.* **91**, 1049-1057 (1987).
- Sager, W. W. & Bleil, U. *Nature* **326**, 488-490 (1987).
- Courtillot, V. & Besse, J. *Science* **237**, 1140-1147 (1987).
- Hargraves, R. B. & Duncan, R. A. *Nature* **245**, 361-363 (1973).
- Gold, T. *Nature* **175**, 526-529, (1955).
- Nakada, M. & Lambeck, K. *Geophys. J. int.* **96**, 497-517 (1989).
- Spada, G., Yuen, D. A., Sabadini, R. & Boschi, E. *Nature* **351**, 53-55 (1991).
- Ricard, Y., Vigny, C. & Froidevaux, C. *J. geophys. Res.* **94**, 13739-13754 (1989).
- Ranalli, G. in *Glacial Isostasy, Sea-level and Mantle Rheology* (eds Sabadini, R., Lambeck, K. & Boschi, E.) 343-378 (Kluwer, Dordrecht, 1991).
- Ricard, Y., Sabadini, R. & Spada, G. *J. geophys. Res.* **97**, 14223-14236 (1992).
- Goldreich, P. & Toomre, A. *J. geophys. Res.* **74**, 2555-2567 (1969).
- Morgan, W. J. in *The Sea*, Vol. 7 (ed. Emiliani, C.) 443-487 (Wiley, New York, 1981).
- Sabadini, R. & Yuen, D. A. *Nature* **339**, 373-375 (1989).
- Richards, M. A. & Hager, B. H. *J. geophys. Res.* **89**, 5987-6002 (1984).
- Ricard, Y., Fleitout, L. & Froidevaux, C. *Ann. Geophys.* **2**, 267-286 (1984).
- Sabadini, R. & Peltier, W. R. *Geophys. J. R. astr. Soc.* **66**, 553-578 (1981).
- Takeuchi, H., Saito, M. & Kobayashi, N. *J. geophys. Res.* **67**, 1141-1154 (1962).
- Spada, G., Yuen, D. A., Sabadini, R., Morin, P. J. & Gasperini, P. *Math. J.* **1**, 65-69 (1990).
- Yuen, D. A., Sabadini, R. & Boschi, E. *J. geophys. Res.* **87**, 10745-10762 (1982).
- Spada, G., Sabadini, R., Yuen, D. A. & Ricard, Y. *Geophys. J. int.* **109**, 683-700 (1992).
- Turcotte, D. L. & Schubert, G. *Geodynamics* (Wiley, New York, 1982).
- Rochester, M. G. & Smylie, D. E. *J. geophys. Res.* **79**, 4948-4951 (1974).
- Gordon, R. G. & Jurdy, D. M. *J. geophys. Res.* **91**, 12389-12406 (1986).
- Kominz, M. A. in *Interregional Unconformities and Hydrocarbon Accumulation* (ed. Schlee, J. S.) Am. Assoc. Petr. Geol. Mem. **36**, 109-127 (Tulsa, 1984).
- Rona, P. A. & Richardson, E. S. *Earth planet. Sci. Lett.* **40**, 1-11 (1978).

ACKNOWLEDGEMENTS. We thank S. Dickman for suggestions. This work was supported by the SCIENCE programme of the EEC.

Mats of giant sulphur bacteria on deep-sea sediments due to fluctuating hydrothermal flow

Jens K. Gundersen*, Bo Barker Jørgensen†, Einer Larsen‡ & Holger W. Jannasch§

* Institute of Biological Sciences, Department of Microbial Ecology, University of Aarhus, 8000 Aarhus C, Denmark

† Max Planck Institute for Marine Microbiology, Fahrenheitstrasse 1, 2800 Bremen 33, Germany

‡ Institute of Biological Sciences, Department of Zoophysiology, University of Aarhus, 8000 Aarhus C, Denmark

§ Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

FILAMENTOUS sulphide-oxidizing bacteria, *Beggiatoa* spp., commonly grow as submillimetre-thin white films on anoxic marine sediments. Unusually thick mats (>1 cm) of giant *Beggiatoa* filaments, 41-120 μm wide and 2-10 mm long, were observed at 2,000 m water depth in the hydrothermal vent fields of Guaymas Basin, Gulf of California¹⁻⁴. We investigated how such dense communities of the largest known bacteria overcome severe diffusion limitation of their substrate supply, and what advantage

they may have by developing such large cell sizes. Oxygen, sulphide, pH and temperature were therefore measured in *Beggiatoa* mats directly on the sea floor. We report here the discovery of small-scale hydrothermal fluid circulations around patches of the bacteria, causing a pulsatory seawater flow into the mats and thereby enhancing the supply of oxygen and sulphide to the bacteria.

A large-scale hydrothermal circulation of sediment pore fluid at the Guaymas Basin is driven by magmatic intrusions below 400-m thick deposits^{5,6}. *Beggiatoa* mats grow around fields of hot, diffuse emanations, visible by shimmering of the overlying water and by black metal sulphide precipitates on the otherwise light-brown sediment. With the aid of the research submersible *ALVIN*, a tripod^{7,8} holding an array of microsensors was carefully positioned over the mats. The data demonstrated micro-environmental conditions very different from those of coastal communities of *Beggiatoa*, where a narrow O₂-H₂S interface within the uppermost 0–1 mm prevails⁹.

The sea water at 2,000 m depth in Guaymas Basin had a temperature of 2.7 °C and was depleted in O₂ to 28 µM, or 8% of atmospheric saturation. Oxygen penetrated irregularly down through several mm of a 20-cm-wide patch of orange mat (Fig. 1). Sulphide of geothermal origin or produced by bacterial sulphate reduction in surface sediment of 3–110 °C^{10,11} was present in concentrations of up to 4,000 µM at 10-cm sediment depth. The H₂S diffused upward to reach the oxic zone at 3–4 mm depth where it was oxidized. Relative to overlying sea water (pH 7.8), the sulphide-rich pore water had a pH minimum of 7.23 at a 10-mm sediment depth. The temperature increased irregularly down through the oxic zone, below which the linear gradient was 10 °C cm⁻¹. The mat surface was exposed to cold bottom water with brief spikes of slightly warmer water (Fig. 2), whereas *Beggiatoa* cells deeper in the 2-cm-thick mat were exposed to <10–20 °C.

Beggiatoa gains energy for chemoautotrophic growth by oxidation of sulphide through elemental sulphur to sulphate^{12,13}. The organisms are highly adapted to grow in steep, opposed microgradients of O₂ from the overlying water and H₂S from

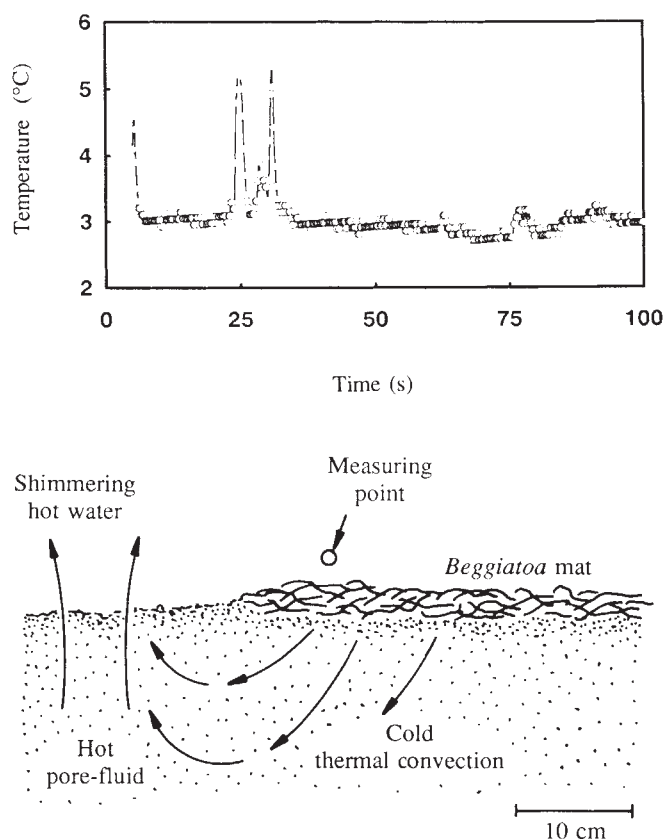


FIG. 2 Series of temperature measurements over 100 s, taken ~1 cm above a *Beggiatoa* mat, indicates the pulsatory flow of sea water and mixing with warm pore-fluid. The small-scale hydrothermal circulation around the mats was deduced from the present study and explains the irregular chemical distributions and development of massive populations of giant, sulphide-oxidizing bacteria.

the sediment below¹⁴. The known species have filament widths of <1 to >30 µm¹⁵. Microscopic examination of collected Guaymas Basin mats showed mostly 40–45 and 120 µm wide and 2–10 mm long *Beggiatoa* filaments, which appeared orange because of the high cytochrome content¹⁶. It has not yet been possible to isolate or grow these giant sulphur bacteria. The upper 0–5 mm of the mats consisted almost solely of living *Beggiatoa* filaments, below which the proportion of dead cell material increased.

From microsensor measurements and mat texture studies of several *Beggiatoa* patches, we derived the hydrothermal flow pattern shown in Fig. 2. As a miniature version of the large-scale hydrothermal flow in Guaymas Basin sediments^{5,6}, the hot emanation appears to draw cold, oxic surface water into the thick *Beggiatoa* mat in an irregular flow. The mud has a crusty or crumbly texture due to precipitated anhydrite and metal sulphides and is thus highly permeable. The pulsatory flow of cold, oxic sea water from above and warm, sulphidic pore-fluid from below increases the supply of both O₂ and H₂S. Much higher quantities of sulphide can thus be oxidized and thicker *Beggiatoa* mats develop than under stagnant conditions where molecular diffusion limits the combined O₂-H₂S supply. The theoretical O₂ flux¹⁷ into the mat just by molecular diffusion would be 2.5 mmol m⁻² day⁻¹, 30-fold lower than into coastal *Beggiatoa* mats⁹. Because the Guaymas mats are 20 times as thick, their O₂ supply would be short >100-fold if limited by molecular diffusion.

We propose the unique small-scale hydrothermal flow at Guaymas Basin vents as the primary reason for massive occurrences of *Beggiatoa*. The coarse mesh of giant filaments provides a strong, permeable texture that accommodates a high substrate

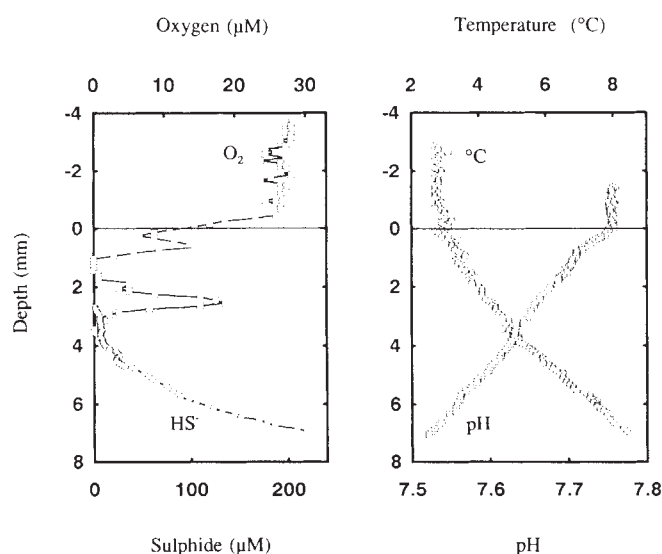


FIG. 1 Chemical zonations through a thick *Beggiatoa* mat and underlying sediment in the hydrothermal fields of Guaymas Basin. Measurements were made *in situ* by the following four microsensors: Clark-type O₂ electrode²¹; Ag/AgS type sulphide electrode¹⁷; glass pH electrode²²; thermistor probe. The microsensors were mounted on a pressure-stable cylinder containing measuring circuits and data acquisition electronics, and on-line communication was established with a portable computer inside the submersible. The cylinder was mounted, gliding accurately on the rails of a tripod^{7,8}, and inserted the microsensors into the sediment where measurements were taken at 50 or 100 µm depth increments.

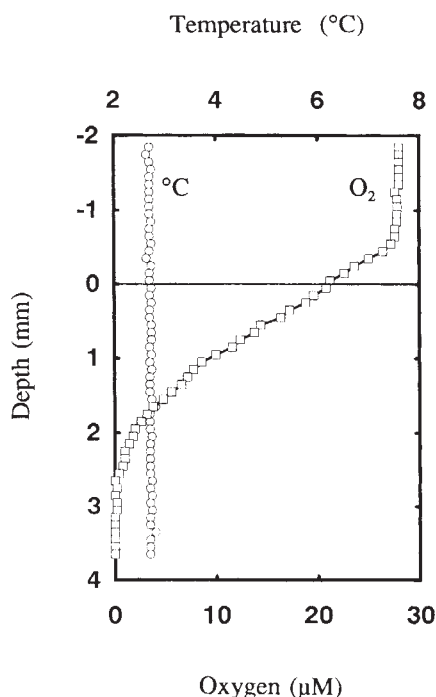


FIG. 3 Typical oxygen and temperature gradients in the non-hydrothermal sediment surrounding the Guaymas Basin vent sites.

supply by the H_2S -containing hydrothermal fluid and the convective flow of oxygenated sea water without the bacteria being swept away¹⁸. By the particular distribution of respiring cytoplasm, which occurs only as a thin film along the outer cell membrane, peripheral to a large central liquid vacuole^{3,4}, the large cells seem to overcome a problem of internal diffusion limitation. The vacuole may furthermore extend the ability of the organisms to survive prolonged exposure to either O_2 or H_2S by its storage capacity for oxidized or reduced substrates.

In sediments surrounding the *Beggiatoa* mats, only 20–50 m away from active vent sites, oxygen penetration to a depth of 2–4 mm showed no irregularities and was comparable to data obtained from eutrophic coastal sediments¹⁹ (Fig. 3). Because of low O_2 concentration in the bottom water, however, O_2 flux into the sediment was only $1.1 \text{ mmol m}^{-2} \text{ day}^{-1}$, typical for continental slope sediments at a few thousand m water depth²⁰. The intensive biomass production by chemosynthetic bacteria and the subsequent organic enrichment of Guaymas Basin hydrothermal sediments are thus locally very restricted to areas of vent emanations. □

19. Rasmussen, H. & Jørgensen, B. B. *Mar. Ecol. Prog. Ser.* **81**, 289–303 (1992).
20. Jørgensen, B. B. in *The Major Biogeochemical Cycles and their Interactions* (eds Bolin, B. & Cook, R. B.) 477–509 (SCOPE, Stockholm, 1983).
21. Revsbech, N. P. *Limnol. Oceanogr.* **34**, 474–478 (1989).
22. Thomas, R. C. *Ion-sensitive Intracellular Microelectrodes, How to Make and Use Them* (Academic, London, 1978).

ACKNOWLEDGEMENTS. We thank F. Hansen, A. Glud, N. P. Revsbech and D. T. Ganzhorn and the Atlantic II/ALVIN crews for their help and the US National Science Foundation, Office of Naval Research and the Danish Environmental Protection Agency for financial support.

Production of sperm reduces nematode lifespan

Wayne A. Van Voorhies

Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721, USA

SEX and death are two fundamental but poorly understood aspects of life^{1–3}. They are often thought to be linked because reproduction requires the diversion of limited resources from somatic growth and maintenance^{4–9}. This diversion of resources in mated animals, often called a cost of reproduction, is usually expressed as a reduction of lifespan in mated animals, although some debate exists on the best way to measure this cost^{4–6,9,10}. I report here that in the soil nematode, *Caenorhabditis elegans*, sex significantly decreases male lifespan without reducing hermaphrodite lifespan. The reduction of mated male lifespan seems to be caused by additional sperm production and not by the physical activity of mating. This conclusion is supported by observations that a mutation reducing sperm production increased mean lifespan by about 65% in both mated males and hermaphrodites. This suggests that spermatogenesis, rather than oogenesis or the physical act of mating, is a major factor reducing lifespan in *C. elegans*. This contradicts the traditional biological assumption that large oocytes are much costlier to produce than small sperm^{11–14}.

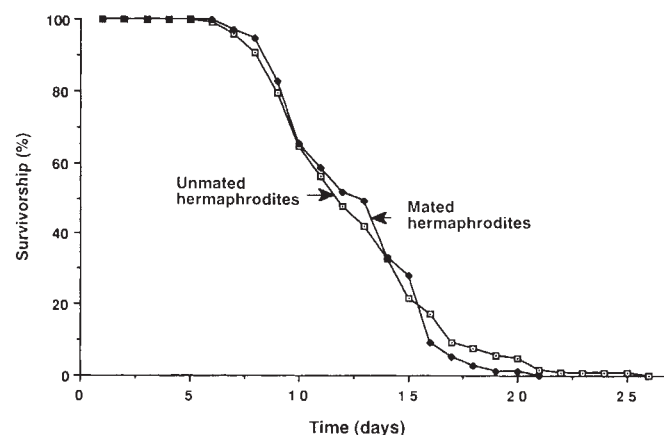


FIG. 1 Survivorship in mated and unmated *C. elegans* hermaphrodites. Lifespans do not vary significantly between mated and unmated hermaphrodites. Lifespans between groups were compared with a Mann-Whitney *U* test. *Z* score (*Z*) = 0.38, *P* = 0.35, sample size (*N*) = 203. Mean lifespan of unmated hermaphrodites = 11.8 days, s.d. = 3.8 days, mean lifespan of mated hermaphrodites = 11.8 days, s.d. = 3.2 days. Age-synchronized populations of the *C. elegans* N2 strain were grown on 3.5-cm Petri dishes containing nematode growth medium (NGM) agar inoculated with *Escherichia coli* (strain OP50)³⁰. N2 is homozygous wild type for all genes with no detectable genetic variability between individuals. Temperature was kept at 20 °C for all experiments. Initial worm density was 25 worms per dish for each group (the mated group contained 10 hermaphrodites and 15 males). Populations were assayed daily for survivorship. To ensure an abundant food supply, worms were transferred daily to fresh dishes (when grown in liquid culture worm lifespan is extended³¹). A worm was scored as dead when it ceased moving and did not respond to a mechanical stimulus.

Received 22 June; accepted 9 October 1992.

1. Jannasch, H. W. in *Hydrothermal Processes at Sea Floor Spreading Centers* (eds Rona, P. A., Boström, K., Laubier, L. & Smith, K. L.) 677–709 (Plenum, New York, 1983).
2. Grassle, J. F. *Science* **229**, 713–717 (1985).
3. Jannasch, H. W., Nelson, D. C. & Wirsen, C. O. *Nature* **342**, 834–836 (1989).
4. Nelson, D. C., Wirsen, C. O. & Jannasch, H. W. *Appl. env. Microbiol.* **55**, 2909–2917 (1989).
5. Einsele, G. J. *et al. Nature* **283**, 441–445 (1980).
6. Lonsdale, P. F. & Becker, K. *Earth planet. Sci. Lett.* **73**, 211–225 (1985).
7. Reimers, C. E. *Deep Sea Res.* **34**, 2019–2035 (1987).
8. Gundersen, J. K. & Jørgensen, B. B. *Nature* **345**, 604–607 (1990).
9. Jørgensen, B. B. & Revsbech, N. P. *Appl. env. Microbiol.* **45**, 1261–1270 (1983).
10. Jørgensen, B. B., Isaksen, M. F. & Jannasch, H. W. *Science* (in the press).
11. Jørgensen, B. B., Zawacki, L. X. & Jannasch, H. W. *Deep Sea Res.* **37**, 695–710 (1990).
12. Nelson, D. C. & Jannasch, H. W. *Arch. Microbiol.* **136**, 262–269 (1983).
13. Nelson, D. C., Jørgensen, B. B. & Revsbech, N. P. *Appl. env. Microbiol.* **52**, 225–233 (1986).
14. Möller, M. M., Nielsen, L. P. & Jørgensen, B. B. *Appl. env. Microbiol.* **50**, 373–382 (1985).
15. Strohl, W. R. in *Bergey's Manual of Systematic Bacteriology* Vol. 3 (eds Staley, J. T., Bryant, M. P., Pfennig, N. & Holt, J. G.) 2091–2097 (Williams & Wilkins, Baltimore, 1989).
16. Prince, R. C., Stokley, K. E., Haith, C. E. & Jannasch, H. W. *Arch. Microbiol.* **150**, 193–196 (1988).
17. Revsbech, N. P. & Jørgensen, B. B. in *Advances in Microbial Ecology* Vol. 9 (ed. Marshall, K. C.) 293–352 (Plenum, New York, 1986).
18. Grant, J. & Bathmann, U. V. *Science* **236**, 1472–1474 (1987).

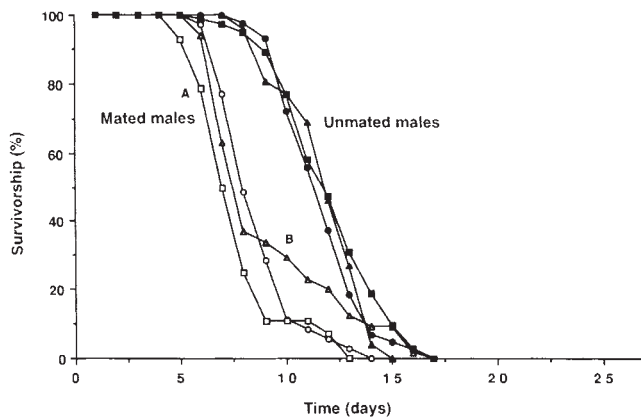


FIG. 2 Survivorship in mated and unmated male *C. elegans*. Mated male lifespan is significantly less than unmated males. $Z=8.0$, $P<0.00005$, $N=217$. Mean lifespan of unmated males = 11.1 days, s.d. = 2.2 days, mean lifespan of mated males = 8.1 days, s.d. = 2.7 days. Data from three different experiments are plotted; two sets of replicates ($\circ$, $\triangle$, $\bullet$, $\blacktriangle$), one group of worms grown in isolation ($\blacksquare$), and a group of males grown with a threefold higher ratio of hermaphrodites ($\square$). Survivorship of males grown in isolation did not differ significantly from other unmated males. There is more variability in mated male survivorship, with one group of worms having an extended 'tail' (B). This tail is probably caused by a low frequency of mating by these males. Male worms placed with a threefold higher ratio of hermaphrodites, (A), show a reduced 'tail length' and decreased lifespan (mean = 6.9 days, s.d. = 2.0, $Z=2.4$, $P<0.001$, $N=28$) relative to the other mated male groups. Numerous (>100) sperm were still visible in 10.5-day-old mated males.

C. elegans' short generation time, ease of culture and well defined genome make it an ideal animal in which to study ageing and the effects of sex on lifespan¹⁵. Natural populations of *C. elegans*, a small, bacteria-eating, roundworm, are almost entirely self-fertilizing hermaphrodites. These hermaphrodites produce and store about 325 sperm before beginning oogenesis. A typical hermaphrodite then produces about 400 oocytes, 80% of which are internally self-fertilized with the hermaphrodite's own sperm¹². Hermaphrodite sperm fertilizes with almost 100% efficiency, making *C. elegans* one of the few animals where progeny number is sperm limited rather than oocyte limited¹⁶. Mating with males (hermaphrodites only mate with males, not with other hermaphrodites) increases hermaphrodite oocyte production two- to threefold relative to unmated worms¹². Males arise in the population from meiotic non-disjunction of the sex chromosomes which occurs in less than 0.5% of hermaphrodite progeny¹⁷. Male *C. elegans* produce and store about 3,000 sperm^{18,19}. Mating induces increased sperm production although the magnitude of the increase is uncertain¹⁹.

To examine the effect of sex on *C. elegans* lifespan I compared survivorship among three groups of worms: (1) all hermaphrodites (unmated hermaphrodites); (2) all males (unmated males); and (3) a combination of males and hermaphrodites (mated males and mated hermaphrodites). Survivorship in each group was plotted as a function of time. Figure 1 shows that mating did not decrease hermaphrodite lifespan, even though mated hermaphrodites produce many more oocytes than unmated hermaphrodites.

Figure 2 shows the great effect of mating on male lifespan. In contrast to hermaphrodites, mating reduces average male lifespan by over 25%. Even a short mating interval significantly reduced male lifespan. Mean lifespan of males that mated only during the first 30 h of their adult life were similar to those of males kept with hermaphrodites their entire life (mean lifespan of 30-h-mated males = 8.9 days, s.d. = 2.1, $N=30$).

Sex is traditionally defined as a mode of reproduction involving the production of haploid gametes and the union of these gametes to form a zygote²⁰. To differentiate which of these

aspects of sex decreased male worm lifespan, I examined the lifespan of worms with a mutation that reduced sperm production. A large number of such spermatogenesis-defective mutations have been isolated in *C. elegans*²¹. Lifespans were compared between two mutant alleles of the *spe-26* gene. These mutants produced apparently normal primary spermatocytes, most of which fail to develop into viable spermatids. Except for reduced sperm production, these mutant worms appear normal in all functions including mating and feeding, with hermaphrodites producing wild-type numbers of oocytes (J. Varkey, manuscript in preparation). If sperm production is a major factor affecting *C. elegans* lifespan these sperm-defective mutants should have increased lifespan and the lifespan of mated mutant males should not be decreased by mating.

Both of these predictions were met. Lifespans of *spe-26* hermaphrodites and males increased an average of 65% over wild-type worms (Figs 3 and 4). Mating did not reduce male lifespan in these mutants. Mating increased mutant male lifespan, although the increase is slight.

Four major results are apparent from these experiments. First, sex significantly reduced male lifespan. Second, sex does not reduce hermaphrodite lifespan. Third, the physical act of mating in itself does not reduce lifespan, a result opposite of that found in *Drosophila melanogaster*^{22,23}. Fourth, mutations in a gene affecting early spermatogenesis greatly increase average lifespan both in hermaphrodites and in mated males.

These results are difficult to reconcile with standard views on the costs-of-reproduction or cost-of-living theories. Both of these theories assume a strong correlation between the energetic costs devoted to reproduction and an organism's lifespan². Traditionally, oocyte production is thought to have a much higher energetic cost than sperm production⁹. This view, based on the small volume of a sperm relative to an oocyte, is thought to translate into a relatively low cost of sperm production. There are examples of animals where males appear to invest large amounts of energy into sperm or seminal fluid production²⁴⁻²⁶. This does not, however, appear to occur in *C. elegans*. The sperm of *C. elegans* are relatively small and produced in limited numbers. A single *C. elegans* oocyte has the same volume as about 500 sperm¹². Thus the 3,000 sperm produced by an unmated

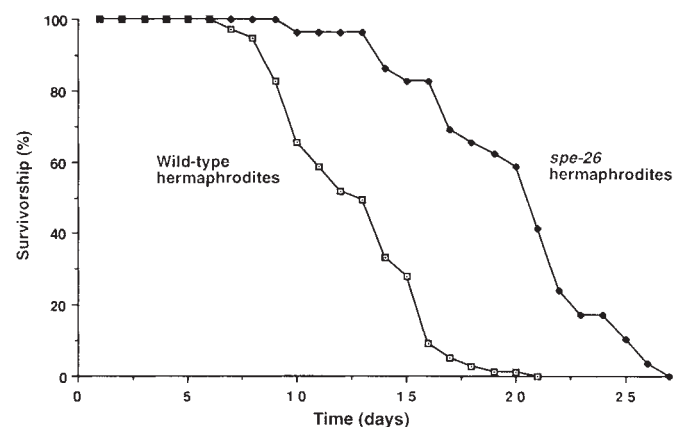


FIG. 3 Survivorship comparison of wild type and *spe-26(hc138ts)* mated hermaphrodites. Lifespan of *spe-26* mutants is significantly greater than wild-type worms. $Z=7.4$, $P<0.00005$, $N=120$. Mean lifespan of *spe-26(hc138ts)* = 19.0 days, s.d. = 4.4 days. Worms identified as *spe-26* have a mutant allele for this gene. At 20 °C this mutant strain is $<10\%$ fertile. Worms with a different *spe-26* allele, *it118ts*, have similar increases in lifespan (mean lifespan mated males 16.1 days, s.d. = 4.0, $N=19$, mean lifespan of mated hermaphrodites 17.6 days, s.d. = 5.4, $N=20$). This indicates that increased lifespan is a property of the *spe-26* gene, not just the function of a single allele. The time of initial egg laying was similar in *spe-26* mutants and wild-type hermaphrodites: *spe-26(it118ts)* = 63.2 h, s.d. = 2.2 h, wild type = 60 h, s.d. = 1.2 h, $N=24$.

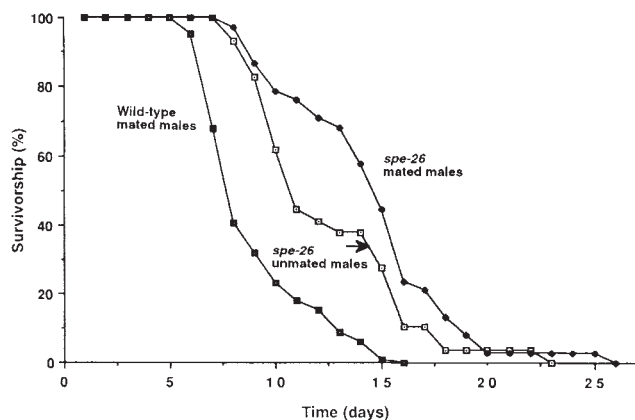


FIG. 4 Survivorship of mated and unmated *spe-26(hc138ts)* males. Lifespans of *spe-26* mated males are significantly greater than wild-type mated males. $Z=6.9$, $P<0.00005$, $N=137$. Mean lifespan of mated males = 13.6 days, s.d. = 3.9 days, $N=37$. Mean lifespan of unmated males = 11.7 days, s.d. = 3.7 days, $N=28$. Nearly 50% of the progeny were male in both mutants, indicating mutant *spe-26* males were successfully mating¹⁶. Mean lifespan in mated *spe-26* males is slightly greater than unmated *spe-26* males. $Z=1.9$, $P=0.03$, $N=67$.

male are equivalent in volume to about 6 oocytes, and the increased sperm production due to mating is equivalent in volume to only a few additional oocytes. Despite this apparently small amount of energy devoted to sperm production, my results show that increased sperm production reduces male lifespan, and reduced sperm production increases worm lifespan. Note that the mutations reported to increase lifespan in *C. elegans* also cause fourfold reductions in *C. elegans* fertility¹⁵, which may be caused by defective sperm production in these mutants²⁷.

Another unexpected result of this study is that mating did not reduce hermaphrodite lifespan. A mated hermaphrodite produces additional oocytes which exceed in number and volume the entire somatic cell content of the animal. Despite this apparently large additional energetic expense, mating did not reduce hermaphrodite lifespan. *C. elegans* lifespan appears to be much more tightly linked to spermatogenesis than oogenesis. This conclusion is further supported by the large increase in lifespan of mutant *spe-26* hermaphrodites and mated males with reduced sperm production.

Life history theory predicts that increased lifespan should be selected for in male worms but not hermaphrodites^{28,29}. By day 10 almost 90% of mated males are dead. Yet mated males of this age still contained hundreds of sperm. Therefore males with increased lifespans could potentially mate later in life and pass on more of their genes before dying. Increasing lifespan in hermaphrodites, in contrast, would not increase their fitness. Unmated hermaphrodites complete egg laying by day 5 and mated hermaphrodites complete egg laying by day 8, long before their average age at death (11.8 days). Thus, hermaphrodites are post-reproductive for a significant portion of their life and there is no selective advantage in their increased lifespans.

Although it appears that increased lifespan should be of selective advantage to male *C. elegans*, laboratory studies must be interpreted with caution. Important ecological factors such as animal density, predation, and food supply were artificially controlled in these experiments. *C. elegans*' reproductive output parameters indicate that they have been selected for rapid reproductive and short generation time¹², traits characteristic of organisms living in unstable environments with a high probability of the organisms dying in any given time. In their natural environment *C. elegans* may die well before reaching their maximum potential lifespan, minimizing the selective pressures for increased lifespans.

A basic assumption of sexual selection and life history theories is that gamete production is generally of minor cost to males. My results show that clearly this is not the case in *C. elegans*. Spermatogenesis rather than oogenesis is the major factor influencing worm lifespan. □

Received 27 July; accepted 9 October 1992.

1. Michod, R. E. & Levin, B. R. (eds) *The Evolution of Sex: An Examination of Current Ideas* (Sinauer, Sunderland, Mass., 1988).
2. Finch, C. E. *Longevity, Senescence and the Genome* (University of Chicago Press, Chicago 1990).
3. Smith, M. J. *The Evolution of Sex* (Cambridge University Press, 1978).
4. Bell, G. & Koufapanou, V. in *Oxford Surveys in Evolutionary Biology* Vol. 3 (eds Dawkins, R. & Ridley, M.) 83–132 (Oxford University Press, 1986).
5. Reznick, D. *Oikos* **44**, 257–267 (1985).
6. Reznick, D. *Trends Ecol. Evol.* **7**, 42–45 (1992).
7. Partridge, L. & Harvey, P. *Nature* **316**, 20 (1985).
8. Hirschfield, M. F. *Ecology* **61**, 282–292 (1980).
9. Calow, P. *Biol. Rev.* **54**, 23–40 (1979).
10. Partridge, L. *Funct. Ecol.* **1**, 317–320 (1987).
11. Giess, M. C., Cazeaux, S. & Murat, M. *Expl. Geront.* **15**, 503–510 (1980).
12. Hodgkin, J. & Barnes, T. M. *Proc. R. Soc. B* **246**, 19–24 (1991).
13. Krebs, J. R. & Davies, N. B. *An Introduction to Behavioural Ecology* (Blackwell, Oxford, 1981).
14. Trivers, R. L. in *Sexual Selection and the Descent of Man* (ed. Campbell, B.) 136–179 (Aldine-Atherton, Chicago, 1972).
15. Johnson, T. E. *Science* **249**, 908–912 (1990).
16. Ward, S. & Carrel, J. S. *Dev. Biol.* **73**, 304–321 (1979).
17. Hodgkin, J., Horvitz, H. R. & Brenner, S. *Genetics* **91**, 67–94 (1979).
18. Ward, S. & Miwa, J. *Genetics* **88**, 285–303 (1978).
19. Wood, W. B. (ed.) *The Nematode Caenorhabditis elegans* (Cold Spring Harbor Laboratory Press, New York, 1988).
20. King, R. C. & Stansfield, W. D. *A Dictionary of Genetics* (Oxford University Press, 1985).
21. L'Hernault, S. W., Shakes, D. C. & Ward, S. *Genetics* **120**, 435–452 (1988).
22. Partridge, L. & Farquhar, M. *Nature* **294**, 580–582 (1981).
23. Partridge, L. & Fowler, K. *Nature* **338**, 760–761 (1989).
24. Boggs, C. L. & Watt, W. B. *Oecologia* **50**, 320–324 (1981).
25. Simmons, L. W. *Nature* **358**, 61–63 (1992).
26. Hennig, W. & Kremer, H. *Int. Rev. Cyt.* **123**, 129–175 (1990).
27. Friedman, D. B. & Johnson, T. E. *Genetics* **118**, 75–86 (1988).
28. Schaffer, W. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3567–3569 (1979).
29. Charlesworth, B. *Evolution in Age-Structured Populations* (Cambridge University Press, 1980).
30. Brenner, S. *Genetics* **77**, 71–94 (1974).
31. Friedman, D. B. & Johnson, T. E. *J. Geront.* **43**, 102–109 (1988).

ACKNOWLEDGEMENTS. This work was done in the laboratory of S. Ward whose entire laboratory staff, particularly J. Varkey and A. Minniti, provided advice and discussion. I also thank L. Abbott, P. Muhlrad, G. Fox, D. Vleck and M. Wojciechowski for reading the original manuscript. This research was supported by grants from the National Institutes of General Medical Sciences to S.W. and a National Science Foundation Research Training Grant.

Viscous fingering of HCl through gastric mucin

K. Ramakrishnan Bhaskar*, Peter Garik†, Bradley S. Turner*, James Douglas Bradley†, Rama Bansil†, H. Eugene Stanley† & J. Thomas LaMont*

* Section of Gastroenterology, University Hospital, Boston University Medical Center, 88 East Newton Street, Boston, Massachusetts 02118, USA

† Center For Polymer Studies and Department of Physics, Boston University, Boston, Massachusetts 02215, USA

THE HCl in the mammalian stomach is concentrated enough to digest the stomach itself, yet the gastric epithelium remains undamaged. One protective factor is gastric mucus, which forms a protective layer over the surface epithelium^{1–4} and acts as a diffusion barrier^{5,6}. Bicarbonate ions secreted by the gastric epithelium⁷ are trapped in the mucus gel, establishing a gradient from pH 1–2 at the lumen to pH 6–7 at the cell surface^{8–10}. How does HCl, secreted at the base of gastric glands by parietal cells, traverse the mucus layer without acidifying it? Here we demonstrate that injection of HCl through solutions of pig gastric mucin produces viscous fingering patterns^{11–18} dependent on pH, mucin concentration and acid flow rate. Above pH 4, discrete fingers are observed, whereas below pH 4, HCl neither penetrates the mucin solution nor forms fingers. Our *in vitro* results suggest that HCl secreted by the gastric gland can penetrate the mucus gel layer

(pH 5–7) through narrow fingers, whereas HCl in the lumen (pH 2) is prevented from diffusing back to the epithelium by the high viscosity of gastric mucin gel on the luminal side.

Viscous fingering^{11–18} is the process by which a complex interface develops when a fluid of lower viscosity is injected into a more viscous one; the driving fluid may penetrate, rather than grossly displace, the stationary fluid, with some of the more viscous fluid remaining behind the interface in lacunae, or 'fjords'. To study viscous fingering, HCl was injected into the solution of mucin in a Hele–Shaw cell^{11,15}, and a highly branched pattern characteristic of viscous fingers was observed (Fig. 1a–c). To confirm that this is indeed viscous fingering, we also did experiments in a flat capillary geometry. The shape of the fingering is dependent on the shape of the cell, and Fig. 1d confirms the usual morphology found in the 'channel' geometry. For further confirmation of viscous fingering phenomena, we studied the reverse flow of (high-viscosity) mucin into (low-viscosity) acid; as expected, the interface is then stable against perturbations and develops symmetrically (Fig. 1e).

We also did fingering experiments at a mucin concentration of 30 mg ml⁻¹, which is the concentration *in vivo*¹⁹ in the pig stomach. At this higher concentration, the viscosity of the mucin solution (41 centipoise (cP)), measured by falling ball microvis-

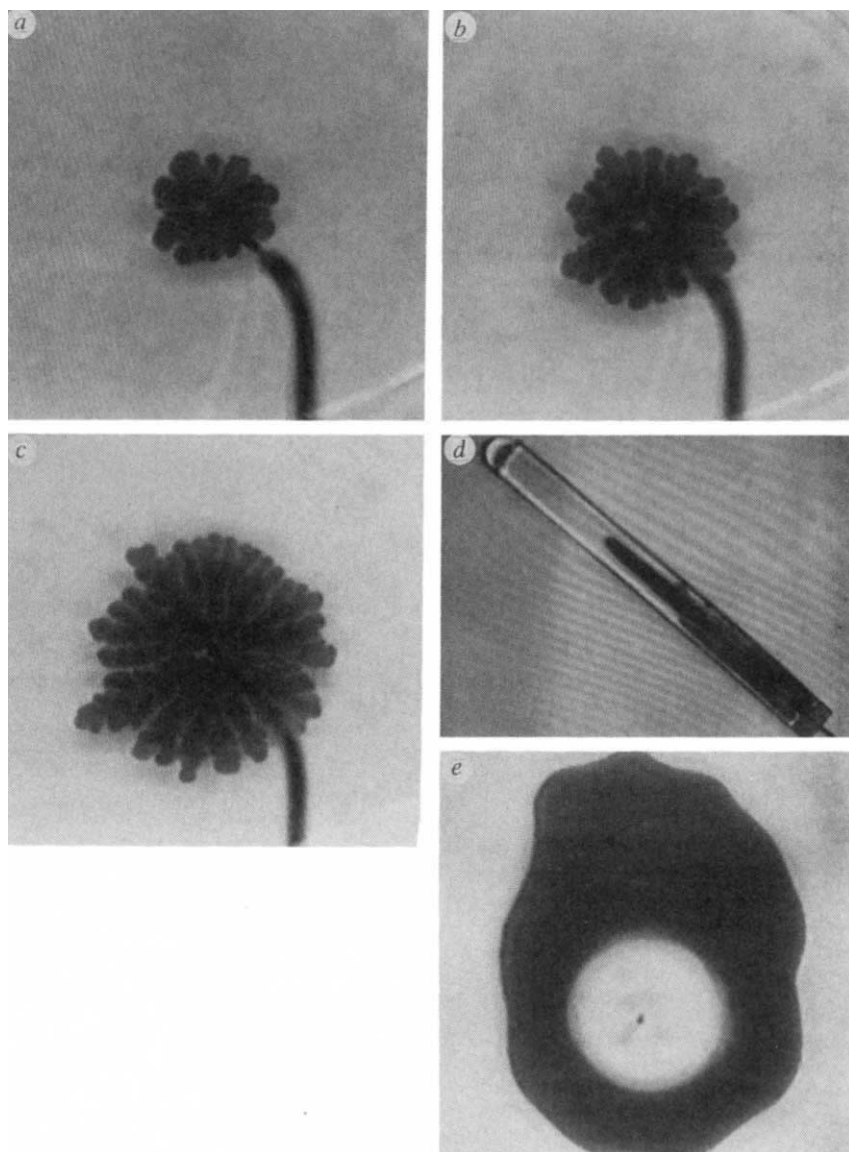
cometry²⁰, is about 12 times that of the 10 mg ml⁻¹ solution (3.6 cP), and we expect non-newtonian viscous effects to be more dramatic. The fingers observed (Fig. 2a) are indeed narrower and more widely spaced than in the 10 mg ml⁻¹ mucin solution (Fig. 1c). This effect becomes more pronounced at high flow rates of acid (Fig. 2b,c), where the tips of the fingers retain their integrity as they propagate: tip-splitting is suppressed. This type of finger, with its apparently parabolic tip, is referred to as 'dendritic' because of its resemblance to the branches which appear during solidification processes (for example, the branches of a snowflake)^{13,14}.

The injection velocity of HCl was $\sim 4 \text{ cm s}^{-1}$ (Fig. 1a–c). Experiments with initial velocities as high as 40 cm s^{-1} revealed that, as fluid velocity increases, finger spacing increases and finger width decreases. In contrast with the lower mucin concentrations and HCl velocities shown in Fig. 1, Fig. 2b,c shows that this trend is followed for a mucin concentration of 30 mg ml⁻¹, and an acid velocity of $\sim 3.5 \text{ cm s}^{-1}$ at a radial distance of 0.5 cm. These parameters are comparable to those *in vivo*.

We also observed viscous fingering with partially purified mucin containing non-mucin proteins and lipids, and thus closer in composition to the mucus gel layer that exists *in vivo*²¹. The

FIG. 1 Viscous fingers formed by HCl (dark) injected into solutions of gastric mucin (white). The patterns were recorded with a video CCD camera (for details see ref. 28). Several frames are shown: a–c, fingering pattern for HCl into mucin (10 mg ml⁻¹, pH 7) at successive time intervals. For tip-splitting fingers, constant injection rate results in a decrease in interfacial velocity with time. The increase in finger packing may be due to this variation. d, Single finger produced by injection of HCl (dark) into mucin (white) in the channel geometry. e, Mucin (white) injected into HCl (dark) does not produce viscous fingers but rather results in complete symmetric displacement. In the experiment illustrated on the cover, HCl without added trypan blue was injected into mucin solution containing the acid indicator dye bromophenol blue, which turns yellow at acidic pH. The absence of colour change indicates that during the passage of HCl, hydrogen ions are confined to the boundary of the fingers and do not diffuse into the mucin solution.

METHODS. The component primarily responsible for the viscoelastic properties of gastric mucus is mucin, a glycoprotein (80% carbohydrate and 20% protein) of large molecular mass ($> 2 \times 10^6$) secreted by the epithelial cells. For our studies we used mucin isolated from pig stomach mucosal scrapings, purified by gel filtration on a Sepharose 2B column followed by density gradient ultracentrifugation in a CsCl gradient according to standard methods²⁹. Viscous fingering experiments were done in a Hele–Shaw cell consisting of two square glass plates (10 cm $\times$ 10 cm) separated by $\sim 300 \mu\text{m}$ and clamped on all four corners. Mucin solution was layered between the plates through a hole in the top plate. HCl (0.1 M), containing 0.04% trypan blue to aid visualization of acid migration, was injected through the top hole (diameter 0.5 mm), using an infusion pump, at a constant rate of volume flow (0.0186 ml s^{-1}). In the absence of dye, turbidity due to aggregation of mucin at low pH²⁰ outlines the finger making it visible, and similar highly branched patterns are observed. Channel flow experiment (d) was done in a flat capillary (5 cm $\times$ 0.4 cm $\times$ 0.04 cm). After mucin solution was aspirated into the capillary, one end was sealed with 'Sealease'. A 30.5 gauge needle was inserted through the seal and acid injected as in the above experiments.



formation of viscous fingers was also strongly dependent on mucin solution pH (Fig. 3). Viscous fingering patterns developed at pH 7 and 5 (Fig. 3a,b), but at pH 4 the bulk of the injected HCl remained on the periphery of the mucin solution and only small, poorly formed fingers were observed (Fig. 3c). In mucin solutions at pH 2, the acid did not enter the mucin at all (Fig. 3d), but instead flowed around the sample, as predicted by our previous observation²⁰ that solutions of gastric mucin undergo profound aggregation and increased viscosity below pH 4, the pH at which viscous fingering begins to disappear.

To simulate the *in vivo* situation where secreted HCl travels toward the lumen through a layer of mucus, we also studied acid migration through a column of mucin solution in a test tube (Fig. 4). When HCl was injected into water alone, it diffused irregularly throughout the tube (upper panel). When HCl was

injected at the same flow rate into a solution of gastric mucin, the acid travelled in a discrete 'channel' to the top, where it then layered horizontally on top of the mucus layer and did not diffuse downward into the solution (lower panel). When simultaneously injected from two syringes, the acid travelled in two discrete 'channels' which remained separate.

On the basis of hydrodynamic theory, viscous fingering is expected when a low-viscosity liquid is driven under pressure into a high-viscosity liquid. Thus viscous fingering offers a physically plausible mechanism whereby 250 ml h⁻¹ of HCl²² can traverse a protective mucus volume of 30–50 ml without causing major disruption of the 500 μ m thick mucus layer²³ between the crypt opening and the gastric lumen. The opossum gastric mucosa²⁴ contains smooth muscle strands and varicose nerve endings near gastric glands, suggesting muscle contraction could give rise to hydrostatic pressure within the crypt during acid secretion. Our results complement the recent *in vivo* observations in rats²⁵, where secreted acid travelled through the mucus layer into the lumen only at restricted regions or channels directly above gastric crypts. We argue that HCl secreted under pressure by gastric glands forms viscous fingers in the mucus gel layer; as the acid passes through the mucus layer it may form a gelled or aggregated mucus boundary around the finger, preventing gross acidification of the surrounding layer of mucus. Such fingers may correspond to the channels suggested in ref. 25. Once free in the lumen, the acid causes the layer of mucin on the luminal side to become more viscous²⁰. In the absence of hydrodynamic pressure on the luminal side, reverse fingering of acid does not occur and passive back-diffusion of acid would be impeded by the high viscosity of this boundary layer.

Although our experiments were not done *in vivo*, we believe there are sufficient supporting data favouring the physiological relevance of our *in vitro* studies of HCl migration through solutions of gastric mucin. The use of mucin is justified because it is the major viscoelastic component of gastric mucus and the higher concentration used in our studies (30 mg ml⁻¹) is comparable to the reported *in vivo* concentration¹⁹. Acid fingers which were relatively thick and closely spaced at 10 mg ml⁻¹ became

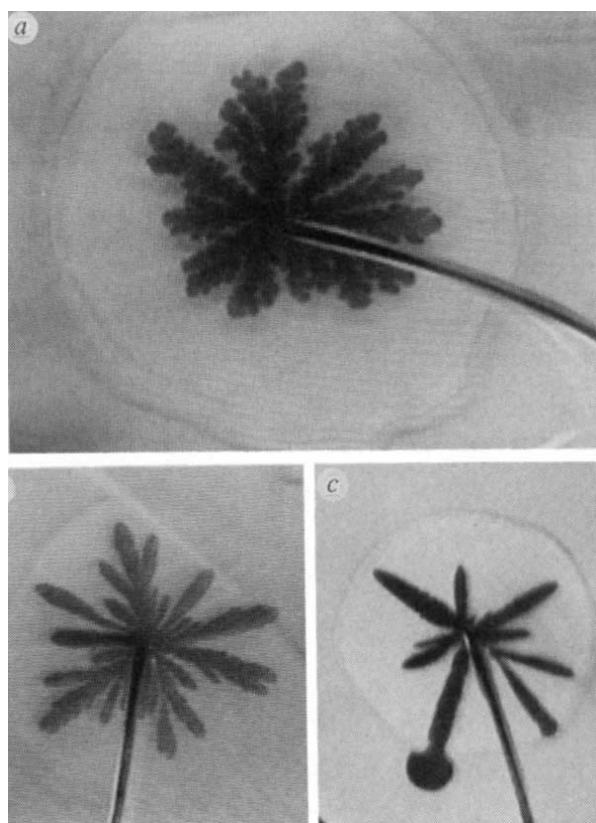


FIG. 2 Viscous fingers formed by HCl (dark) injected into 30 mg ml⁻¹ solutions of mucin (white). Effect of increasing acid flow rate and temperature: a, injection at 0.0186 ml s⁻¹, results in narrower fingers, the fjords, white areas between the dark acid fingers, are more prominent, indicating less disturbance to the mucin layer; b, for same mucin concentration as in a, injection of HCl at 0.33 ml s⁻¹ results in thinner, more widely spaced fingers; c, same experiment as b done with both mucin solution and acid at 37 °C (other data shown were taken at ambient room temperature).

METHODS. To estimate the HCl secretion velocity at the mouth of the crypt, we assume Poiseuille flow, and approximate the crypt as a cylinder of length $l=1,000 \mu\text{m}$, and radius $r=20 \mu\text{m}$ ³⁰. The pressure in the middle of the rat crypt was measured by Agren and Holm³¹ as 19 mm Hg in the resting state, and 22 mm Hg after pentagastrin stimulation. These authors report a relative pressure in the lumen of 0 mm; others³² have reported the luminal pressure as 5 mm Hg. For simplicity, we assume that at rest a 14–19 mm pressure drop occurs across the mucin layer as opposed to a muscular constriction of the neck of the gland. Such a tensile load is below the critical yield stress of mucin³³. The excess pressure gradient G after stimulation is then about 3 mm Hg per 500 μm . From the Poiseuille formula, and using our measured viscosity of HCl of $\eta=1.0 \text{ cP}$, the mean flow velocity is $Ga^2/8\eta=4 \text{ cm s}^{-1}$. This acid velocity agrees roughly with the $\sim 1 \text{ cm s}^{-1}$ flow velocity calculated using the model crypt dimensions of $1.3 \times 10^{-6} \text{ ml}$, and the frequency of pressure oscillation (0.1 Hz) also reported by Agren and Holm³¹.

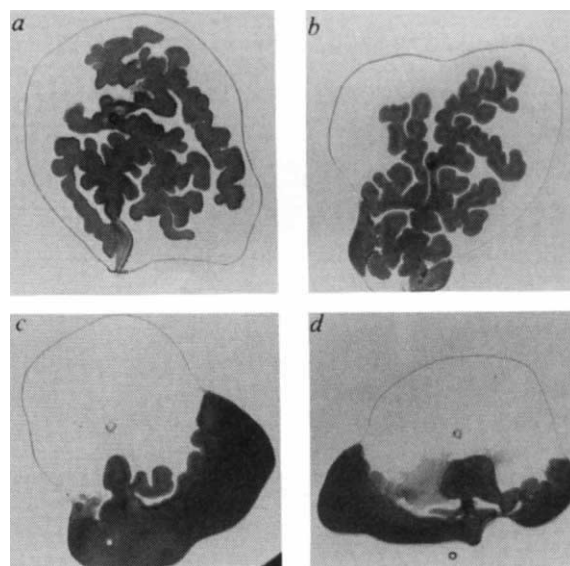
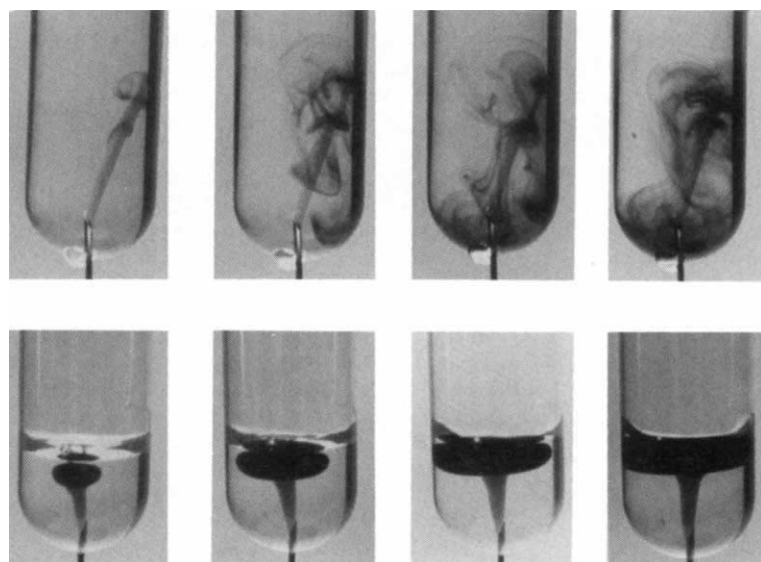


FIG. 3 Viscous fingering of HCl in gastric mucin, where the injection is now from the periphery (as opposed to the central hole). Effect of varying pH of mucin solution. Acid-formed fingering patterns in mucin solutions at a, pH 7 and b, pH 5. In striking contrast, acid barely penetrated mucin at c, pH 4 or d, pH 2.

METHODS. Mucin, 10 mg ml⁻¹ in 10 mM phosphate buffer. HCl (0.1M) containing 0.04% trypan blue injected at the same flow rate in all four experiments. Partially purified mucin obtained by Sepharose 2B gel filtration was used.

FIG. 4 Lower panel, HCl injected into a column of gastric mucin of concentration 2.5 mg ml^{-1} in 0.1 M NaCl , pH 6.5. HCl (flow rate 0.01 ml s^{-1}) forms a discrete channel. The four pictures show the channel at successive increasing time intervals (left to right). Upper panel, Control experiment with water under the same conditions shows the rapid diffusion of acid.

METHODS. Acid (0.1 M) containing trypan blue (0.04%) injected through a needle pierced into the bottom of the tube containing mucin solution. Flow rate controlled using an infusion pump.



more distinct and widely spaced on increasing mucin concentration to 30 mg ml^{-1} . Thus the studies at lower mucin concentrations served to establish the trend in viscous fingering morphology and suggest that if the *in vivo* concentration were greater than 30 mg ml^{-1} , the fingers would be even finer and less disruptive of the mucus layer. Ever since Heatley³ proposed the barrier theory of gastric mucus in 1959, the question of whether mucus represents an effective barrier or not has been the subject of considerable investigation. Although the ability of the mucus gel layer to maintain a pH gradient is a subject of controversy^{26,27}, the notion that gastric mucus impedes the diffusion of H^+ has been supported by many *in vitro* and *in vivo* experiments^{5,6,8}. Our *in vitro* studies offer a physical-chemical basis for the barrier function of gastric mucus *in vivo*. □

Possible blindsight in infants lacking one cerebral hemisphere

Oliver Braddick*, Janette Atkinson*, Bruce Hood*, William Harkness†, Graeme Jackson† & Faraneh Vargha-Khadem†

* Visual Development Unit, Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK

† Hospital for Sick Children and Institute for Child Health, Great Ormond Street, London WC1, UK

PATIENTS with damage to the striate cortex have a subjectively blind region of the visual field, but may still be able to detect and localize targets within this region^{1,2}. But the relative roles in this 'blindsight' of subcortical neural systems, and of pathways to extra-striate visual areas, have been uncertain³. Here we report results on two infants in whom one cerebral hemisphere, including both striate and extra-striate visual cortex, needed surgical removal in their first year. Single conspicuous targets in the half-field contralateral to the lesion could elicit fixations, implying detection and orienting by a subcortical system. In contrast, binocular optokinetic nystagmus (OKN), for which a subcortical pathway has often been thought adequate, showed a marked asymmetry. In normal neonates, fixation shifts and OKN have both been taken to reflect subcortical control⁴; our results are consistent with subcortical control for fixation but not for OKN.

Hemispherectomy surgery, to relieve intractable myoclonic seizures caused by congenital unilateral megalencephaly (Fig. 1), was performed on infant P.P. (right hemisphere) at 4 months of age and on infant L.A.H. (left hemisphere) at 8 months. The complete removal of one hemisphere was confirmed by the neuropathologist's examination of removed tissue in each case. The tests we report followed these operations by 3–7 months (P.P.) and 8–10 months (L.A.H.).

In the week before surgery, both infants (then heavily medicated to alleviate their seizures) showed very poor visual behaviour. P.P. showed no consistent shifts of gaze to a target; L.A.H. showed some visually guided reaching but poor shifts of gaze, and acuity around 1 cycle deg^{-1} (equivalent to a newborn). But in postoperative testing, both children were visually alert with full eye movements, although each showed intermittent strabismus. Their visual acuity was in the normal range for 11–15 month infants ($8\text{--}12 \text{ cycle deg}^{-1}$, tested by preferential

Received 10 June; accepted 22 October 1992.

- Allen, A. in *Physiology of the Gastrointestinal Tract* Vol. 1 (eds Johnson, L. R. et al.) 617–639 (Raven, New York, 1981).
- Hollander, F. *Arch. Intern. Med.* **93**, 107–120 (1954).
- Heatley, N. G. *Gastroenterology* **37**, 313–317 (1959).
- Allen, A. *Trends Biochem. Sci.* **8**, 169–173 (1983).
- Williams, S. E. & Turnberg, L. A. *Gastroenterology* **79**, 299–304 (1980).
- Pfeiffer, C. *Am. J. Physiol.* **240**, G176–182 (1981).
- Flemström, G. in *Physiology of the Gastrointestinal Tract* 2nd edn (eds Johnson, L. R. et al.) 1011–1029 (Raven Press, New York, 1987).
- Williams, S. E. & Turnberg, L. A. *Gut* **22**, 94–96 (1981).
- Takeuchi, K. et al. *Gastroenterology* **84**, 331–340 (1983).
- Allen, A. & Garner, A. *Gut* **21**, 249–262 (1980).
- Hele-Shaw, H. S. *Nature* **58**, 34–36 (1898).
- Nittmann, J., Daccord G. & Stanley, H. E. *Nature* **314**, 141–144 (1985).
- Ben-Jacob, E. & Garik, P. *Nature* **343**, 523–530 (1990).
- Buka, A., Kertész, J. & Vicsek, T. *Nature* **323**, 424–425 (1986).
- May, S. E. & Maher, J. V. *Physical Rev.* **A40**, 1723–1726 (1989).
- Joseph, D. D. *Eur. J. Mech. Fluids* **B9**, 565–596 (1990).
- Garik, P. et al. *Physical Rev. Lett.* **66**, 1606–1609 (1991).
- Fabry, T. L. *Gastroenterology* **98**, A42 (1990).
- Allen, A., Pain, R. H. & Robson, T. R. *Nature* **264**, 88–89 (1976).
- Bhaskar, K. R. et al. *Am. J. Physiol.* **261**, G827–G832 (1991).
- Murthy, V. L. N., Sarosiek, J., Slomiany, A. & Slomiany, B. L. *Biochem. biophys. Res. Commun.* **121**, 521–529 (1984).
- Makhlouf, G. M. in *Physiology of the Gastrointestinal Tract* Vol. 1, (eds Johnson, L. R. et al.) 551–566 (Raven, New York, 1981).
- Kerss, S., Allen, A. & Garner, A. *Clin. Sci.* **63**, 185–188 (1982).
- Seelig, L. L. et al. *Am. J. Anat.* **174**, 15–26 (1985).
- Holm, L. & Flemström, G. *J. int. Med.* **228**, (suppl) 91–95 (1990).
- Patrónella, C. K., Vanek, I. & Bowen, J. C. *Gastroenterology* **95**, 612–618 (1988).
- Wallace, J. L. *Am. J. Physiol.* **256**, G31–G38 (1989).
- Lal, J. & Bansil, R. *Physica A* **186**, 88–96 (1992).
- Gong, D. et al. *Am. J. Physiol.* **259**, G681–G686 (1990).
- Bloom, W. & Fawcett, D. W. *A Textbook of Histology* 8th edn 433 (W. B. Saunders, Philadelphia, 1966).
- Agren, J. & Holm, L. *Acta physiol. scand.* **140**, 36A (1990).
- Castell, D. O. in *Esophageal Motility Testing* (eds Castell, D. O. et al.) 21 (Elsevier, New York, 1987).
- Sellers, L. A. et al. *Biorheology* **24**, 615–623 (1987).

ACKNOWLEDGEMENTS. We thank S. Pajevic for technical assistance and D. Stauffer for reading the manuscript. This work was supported by funds from the NIH, NSF and ONR.

looking⁵). On informal testing each child ignored a toy presented in the half-field contralateral to the removed hemisphere, but promptly reached for the same toy in the ipsilateral half-field.

In our fixation shift procedure^{6,7}, the infant initially sees a screen containing a central flashing face-like target, which is replaced by a contrast-reversing target 23 deg either right or left of centre. The direction and latency of the initial fixation shift is recorded. A number of control trials, in which the central target was followed by a blank screen, were included in the random sequence.

With binocular viewing, P.P. made initial shifts of gaze in the correct direction to 100% (7/7) of targets presented in the right half-field, and 82% (9/11) in the left half-field whose cortical projection is missing. Corresponding results for L.A.H. were 88% (7/8) in the left half-field and 100% (16/16) in the decorticate right half-field. Thus both infants show performance considerably above chance (binomial $P < 0.05$) for targets that could not produce any visual cortical representation. P.P.'s responses had significantly longer latencies for the half-field contralateral to the lesion (mean = 1.84 s, s.d. = 1.67) compared to the good field (mean = 0.47 s, s.d. = 0.16; independent groups $t = 2.15$, $P = 0.05$), but there was no significant difference for L.A.H. (affected field: mean = 0.612 s, s.d. = 0.41; good field: mean = 0.48, s.d. = 0.22). In control trials, P.P. made 5/11 initial fixations towards the decorticate half field, whereas L.A.H. made 3/10. Thus the results cannot be explained by a bias in looking towards the decorticate half-field in the absence of detectable stimulation.

Different aspects of these results were explored further in each infant. For L.A.H., we repeated the test for each eye viewing separately. With the right eye she could respond to targets in both half-fields (left, 12/12 correct; right, 7/7) as in binocular viewing. But the left eye yielded significantly poorer performance (2/16) in the right half-field, although still good (11/11) in the left. This pattern of results suggests that no uncrossed pathway from the left eye to the damaged left side of the brain is functional to elicit fixation shifts.

With P.P., shifts of fixation were compared between a 'compe-

tition' condition, in which the central target remained visible, and the 'non-competition' condition where it disappears at the onset of the lateral target⁷. In the non-competition condition P.P. made fixation shifts to the target in his left half-field, but competition reduced this performance much more severely than in the right half-field (Fig. 2). Such competition effects normally occur in both half-fields in the first month of life, but significantly reduce by 3 months, a change linked to cortically mediated attentional mechanisms^{6,7}. Qualitatively, infant P.P. often appeared to blink and make nodding head movements in order to 'unlock' his central fixation and move his gaze to a target in his defective half-field.

With binocular stimulation of OKN, both infants showed marked asymmetry, with brisk responses to stimulus movement in the direction towards the decorticate half-field (that is, contralateral-directed with respect to the damage). In the direction towards the good half-field, the only response, if any, was

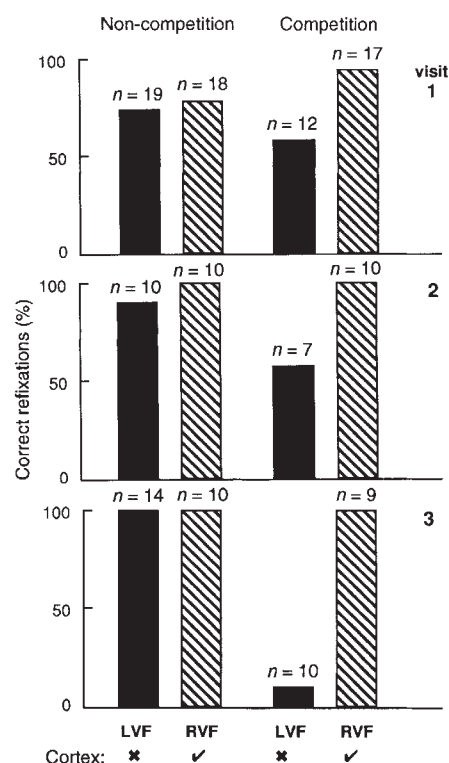


FIG. 2 Comparison of 'competition' and non-competition conditions for fixation shifts in subject P.P. When the observer is satisfied that the infant is fixating the initial high-contrast (88%) central stimulus, he initiates presentation of a lateral target at 23 deg either right or left of centre. Target is one cycle of 0.08 cycle deg⁻¹ vertical square-wave grating in a strip 12 × 32 deg, contrast 50%, matched in mean luminance (8 cd m⁻²) to the grey background, reversing in contrast 6 times per second. In non-competition trials the central target disappears at this point; in competition trials it remains visible. The observer is 'blind' to target location of the target and makes a forced-choice whether the initial fixation shift is to right or left. Latency is subsequently measured from a time-coded videotape record of head and eye movements. (Initial results on both infants reported in the text used the same procedure but with non-competition trials only and a target contrast of 88%). Bars show the percentage of correctly directed fixations to targets appearing in the left (LVF) and right (RVF) visual fields, on three successive visits. Visit 1, age 8 months, 3 months after right hemispherectomy; Visit 2, age 10 months (5 months post-operative); Visit 3, 12 months, (7 months post-operative). The left visual field would normally project to the hemisphere which is absent in this patient; *n* above each bar refers to the total number of trials with that target. Whether the apparent trend of decreased responses in the competition condition across the three successive visits represents a long-term behavioural change is an important question for further studies.

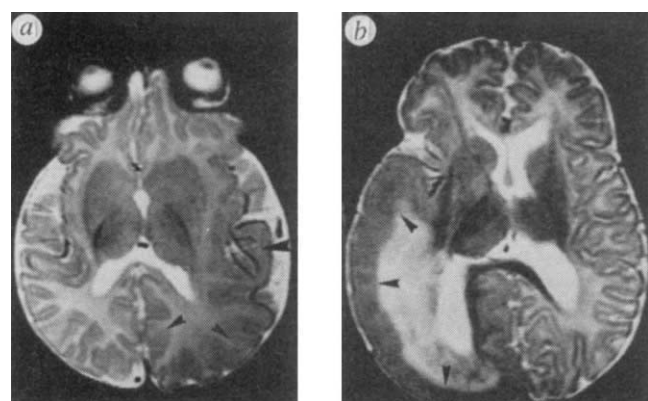
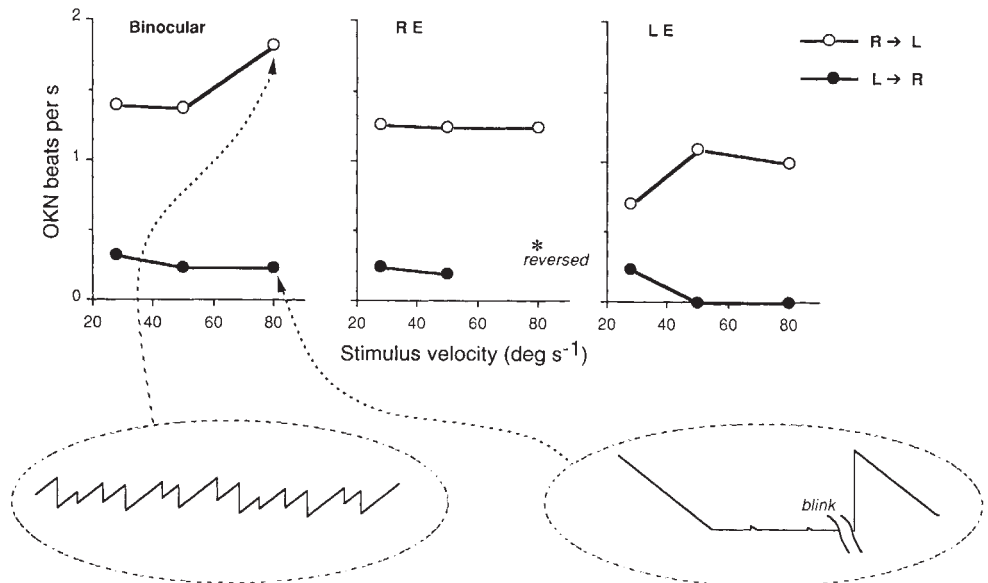


FIG. 1 Pre-operative magnetic resonance images of the two infants' brains (T2-weighted axial images). *a*, Patient P.P.: section at level of the thalamus and basal ganglia shows right occipital region (shown on right) lacking grey/white matter differentiation resulting in apparent thickening of the grey matter (small arrows). Anteriorly large gyri appear, with decreased signal throughout the hemisphere (large arrow). Histology after hemispherectomy showed an extensive neuronal migration defect with polymicrogyria mainly in the area supplied by the middle cerebral artery. *b*, Patient L.A.H.: section at level of basal ganglia. The right hemisphere (shown on right) appears normal. Abnormal morphological changes in the left hemisphere include absence of gyri, thickening of cortex, and marked signal abnormality in the remaining white matter. These are most prominent in parietal and occipital lobes (arrows). Pathological examination after hemispherectomy of the resected specimen showed pachygyria with nodular heterotopia and superficial myelination.

FIG. 3 The asymmetry of optokinetic nystagmus for subject P.P. tested at age 9 months (5 months after R hemispherectomy). To stimulate OKN, infants were seated in front of a rear-projection screen (subtending 120° wide × 100° high) on which a moving film strip projected a texture of irregular high-contrast blobs, moving at speeds of 28–80 deg s⁻¹ either leftwards or rightwards. Beats per second derived from an observer counting fast-phase eye movements over a 10–20 s period. In binocular right-eye and left-eye viewing conditions, brisk OKN is elicited by right-to-left motion; for left-to-right motion the number of beats is very small and does not represent a normal OKN response (the traces at bottom illustrate the observer's description of the pattern of eye movements in the two directions). RE, right eye; LE, left eye.



occasional long tracking movements or reversed OKN. Figure 3 shows results for P.P. Over the whole velocity range tested, he shows the asymmetry for stimulation of each eye individually, as well as binocularly; note that this is quite different from the pattern seen in very young normal infants⁸ and subjects with defective binocularity^{8,9}, who show no temporal-directed response in either eye but no evident asymmetry in binocular viewing. Normal infants over 3 months, and adults, show very similar OKN responses to nasal-directed and temporal-directed motion of these stimuli⁸. L.A.H. showed little response in either direction with her right eye (contralateral to the absent hemisphere), and only nasal-directed response with her left eye.

The nucleus of the optic tract (NOT) on each side of the pretectum is believed to control OKN responses to stimuli moving towards that side^{10,11}. The nasal-directed response in young infants has been widely interpreted as reflecting a functional crossed pathway, direct to the NOT, with the temporal-directed response depending on later maturation of an uncrossed pathway through binocular cortical neurons. But if a direct crossed pathway was functional in these patients, it should allow nasal-directed responses in both eyes, which were not seen. The results imply that in these patients, OKN in either direction requires an intact pathway through the visual cortex. P.P., but not L.A.H., was able to use an uncrossed pathway on the intact side; this may possibly reflect differences in cortical binocularity between the two infants. A similar pattern to that of P.P. has been reported¹² for a child with a large cyst in one hemisphere. Overall, our results are consistent with proposals^{13,14} that OKN in human adults is under cortical control for both directions. Cortically blind patients have often been reported to lack OKN, although the response has been found to recover after some months¹⁵. Developmentally, the results suggest either that a cortical pathway operates in normal newborns, or that an initially functional route direct to the NOT drops out in favour of cortical control at a very early stage.

The fixation shift results make it clear that orienting behaviour can be evoked by stimuli that cannot have been processed by any contralateral cortical structures. The relation of this behaviour to 'blindsight' is debatable because the infants cannot report their subjective experience. We presume that the behaviour depends on subcortical pathways involving the superior colliculus, although given the early abnormalities of these brains, anomalous projections to the hemisphere ipsilateral to the stimulus cannot be excluded. The results support earlier findings with older hemispherectomy patients^{16,17} and are consistent with models of subcortical control of visual orienting in

normal newborns^{4,18}. The presence of these responses in hemispherectomized infants as young as 8 months and within 3 months of surgery, argues against any need for long-term plasticity, or acquisition of a special strategy, in order to use this subcortical route. There may, of course, have been shorter-term adaptations first to abnormality and then to absence of the cortex on one side.

The monocular results imply that the crossed pathway is necessary for an orienting response to the decorticate half-field. This is somewhat surprising given primate evidence that the colliculus receives substantial uncrossed input¹⁹. But in hemianopic patients, only the crossed pathway can convey target inhibition from the 'blind' field that delays a saccade towards the intact field²⁰, and the crossed pathway also appears dominant in fixation control early in development²¹.

A persisting central stimulus can largely suppress the orienting response, suggesting that cortically based mechanisms, when present, are prepotent in competition with the subcortical orienting mechanism. Generally, the study of hemispherectomized children shows that cortical and subcortical visual systems interact in different ways for different visual functions, and raises the important question of how far these interactions may be plastic during early development. □

Received 13 July; accepted 22 October 1992.

1. Pöppel, E., Held, R. & Frost, D. *Nature* **243**, 295–296 (1973).
2. Weiskrantz, L. *Blindsight, a Case Study and Implications* (Oxford Univ. Press, Oxford, 1987).
3. Cowey, A. & Stoerig, P. *Trends Neurosci.* **14**, 140–145 (1991).
4. Atkinson, J. *Hum. Neurobiol.* **3**, 61–74 (1984).
5. Mohn, G. & van-Hof van Duin, J. *Doc. ophth. Proc. Ser.* **45**, 363–372 (1986).
6. Hood, B. & Atkinson, J. *Dev. Med. Child Neurol.* **32**, 1067–1077 (1990).
7. Atkinson, J., Hood, B., Braddick, O. & Wattam-Bell, J. *Perception* (in the press).
8. Atkinson, J. in *Developmental Neurobiology of Vision* (ed. Freeman, R. D.) (Plenum, New York, 1979).
9. Crone, R. A. *XIIIth ISCEERG Symposium, Doc. ophth. Proc. Ser.* **45**, 9–18.
10. Hoffmann, K. P. & Schoppmann, A. *Brain Res.* **99**, 359–366 (1975).
11. Hoffmann, K. P. *Prog. Brain Res.* **80**, 173–182 (1989).
12. Van Hof-van Duin, J. & Mohn, G. *Behav. Brain Res.* **10**, 163–175 (1983).
13. Mehdorn, E. *Invest. Ophth. vis. Sci.* **24** (suppl.), 23 (1983).
14. Harris, L. Lewis, T. L. & Maurer, D. *Exp. Brain Res.* (in the press).
15. ter Braak, J. W. G., Schenk, V. W. D. & van Vliet, A. G. M. *J. Neurol. Neurosurg. Psychiatr.* **34**, 140–147 (1971).
16. Perenin, M. T. & Jeannerod, M. *Neuropsychologia* **16**, 1–12 (1978).
17. Ptilo, A., Lassonde, M., Lepore, F. & Ptilo, M. *Neuropsychologia* **25**, 869–879 (1987).
18. Bronson, G. W. *Child Dev.* **45**, 873–890 (1974).
19. Schiller, P. in *Handbook of Physiology, The Nervous System III Sensory Processes Part 1*. (ed. Darian-Smith, I.) 457–505 (American Physiological Society, Bethesda, 1984).
20. Refai, R., Smith, J., Krantz, J., Cohen, A. & Brennan, C. *Science* **250**, 118–121 (1990).
21. Lewis, T. L., Maurer, D. & Blackburn, K. *Vision Res.* **25**, 943–950 (1985).

ACKNOWLEDGEMENTS. We thank J. Wattam-Bell, F. Weeks and S. Anker for assistance and collaboration in vision testing; C. E. Polkey, H. Cross, F. Kirkham and J. Wilson for their help in clinical aspects of work with these patients; the families of the infant subjects; and L. Weiskrantz, A. Cowey, T. Lewis, L. Harris and H. Barlow for comments. This work is supported by the Medical Research Council.

Cloning and expression of a rat brain L-glutamate transporter

Gilia Pines*, Niels C. Danbolt†, Magnar Bjørås‡§, Yumin Zhang*, Annie Bendahan*, Lars Eide‡§, Hermann Koepsell||, Jon Storm-Mathisen†, Erling Seeberg‡§ & Baruch I. Kanner*¶

* Department of Biochemistry, Hadassah Medical School, The Hebrew University, PO Box 1172, 91010 Jerusalem, Israel

† Anatomical Institute, University of Oslo, PO Box 1105, Blindern, N-0137 Oslo, Norway

‡ Biotechnology Centre, University of Oslo, PO Box 1125, Blindern, N-0136 Oslo, Norway

§ Division for Environmental Toxicology, Norwegian Defence Research Establishment, PO Box 25, N-2007 Kjeller, Norway

|| Max-Planck-Institut für Biophysik, Kennedy Allee 70, 6000 Frankfurt-am-Main 70, Germany

SYNAPTIC transmission of most vertebrate synapses is thought to be terminated by rapid transport of the neurotransmitter into presynaptic nerve terminals or neuroglia¹⁻⁵. L-Glutamate is the major excitatory transmitter in brain and its transport represents the mechanism by which it is removed from the synaptic cleft and kept below toxic levels^{5,6}. Here we use an antibody against a glial L-glutamate transporter from rat brain⁷ to isolate a complementary DNA clone encoding this transporter. Expression of this cDNA in transfected HeLa cells indicates that L-glutamate accumulation requires external sodium and internal potassium and transport shows the expected stereospecificity. The cDNA sequence predicts a protein of 573 amino acids with 8-9 putative transmembrane α -helices. Database searches indicate that this protein is not homologous to any identified protein of mammalian origin, including the recently described superfamily of neurotransmitter transporters. This protein therefore seems to be a member of a new family of transport molecules.

The L-glutamate transporter cDNA was isolated by immunoscreening of a λ zap library from rat brain⁸ with an antibody raised against the purified transporter, recently shown to be of glial origin^{7,9}. A fragment of the cDNA of one of the immunopositive clones, containing the presumed carboxyl terminus of the transporter, was used to screen another λ zap library with inserts of ≥ 4 kilobases (kb) (refs 10, 11). One of the clones, pT7-GLT-1, was found to direct the expression of Na⁺-dependent L-[³H]glutamate transport upon transfection of HeLa cells infected with the vaccinia/T7 recombinant virus¹²⁻¹⁴ (Fig. 1a). In the absence of Na⁺, low levels of transport were detected which were similar to those observed when pT7-GAT-1 was expressed with or without Na⁺. This latter plasmid encodes the GABA_A transporter¹⁰. The nature of the residual uptake is not known, but possibly represents translocation through a transport system endogenous to HeLa cells. The sodium-dependent L-glutamate transport evoked by the pT7-GLT-1 plasmid was stereospecific, which is a well-known property of this system^{3,9}. It was strongly inhibited by 100 μ M of L-glutamate but not by the same concentration of D-glutamate (data not shown). The apparent K_m in intact cells was significantly higher (10 μ M) than in membrane preparations¹⁵ or in the purified and reconstituted transporter⁹, possibly as a result of endogenous L-glutamate diluting the radiolabelled tracer. A reconstituted system derived from HeLa cells was developed, allowing us to clarify this issue and to verify the requirement of the influx for internal potassium¹⁵⁻¹⁷. The transport system has three substrates: Na⁺, L-glutamate and K⁺ (refs. 4, 5, 15-17); physiologically, it takes up the first two and extrudes K⁺ in an electrogenic process (positive charge moving inwards). The reconstituted system permits the removal of endogenous substances, as well as manipulation of internal ion composition. Transport in this

system was completely Na⁺-dependent and effectively inhibited by L-glutamate but not by D-glutamate (Table 1). Furthermore, other neurotransmitters do not seem to interact with the system because there was essentially no inhibition with unlabelled GABA, dopamine, noradrenaline and serotonin (Table 1). The apparent K_m was $\sim 2 \mu$ M (Fig. 1b), in reasonable agreement with the literature¹⁵⁻¹⁷. Several of the substrate analogues defining the pharmacological profile of glutamate transport in the central nervous system are also competitive inhibitors of the activity encoded by pT7-GLT-1 cDNA (K_i values in parentheses, in μ M: L-aspartate (0.2); D-aspartate (0.9); cysteine sulphinate (1.7); threo-3-hydroxy-DL-aspartate (1.0); and L-trans-pyrrolidine-2,4-dicarboxylate (0.73) (see also Fig. 1b)). The latter is a glutamate analogue that inhibits transport of L-glutamate but does not prevent it from binding to its receptors¹⁸. Two other analogues, dihydrokainate and L-amino adipate, selectively inhibit L-glutamate transport in different brain areas^{19,20}. Both compounds inhibit the activity encoded by pT7-GLT-1 (Table 1). There is an essentially complete dependence on internal K⁺ with or without the K⁺-specific ionophore valinomycin, and in the presence of the highly permeant thiocyanate anion in the external medium (Table 1). This indicates that the dependence of the transport process on internal potassium is not simply due to its ability to create an internally negative membrane potential and thereby stimulate this electrogenic process^{9,15}. The permeant thiocyanate anion would also be expected to set up a membrane potential of this polarity, yet fails to drive the process in the absence of internal potassium. Thus, this expressed transporter genuinely mimics all known properties of the (Na⁺ + K⁺)-coupled L-glutamate transporter from rat brain.

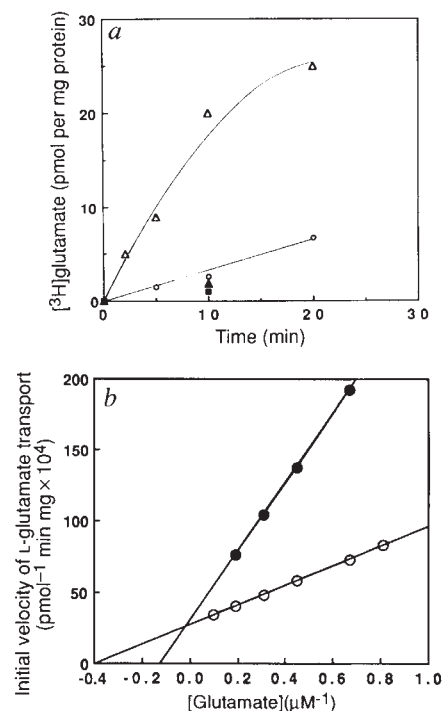


FIG. 1 Transport of L-[³H]glutamate in HeLa cells transfected with GLT-1 cDNA. HeLa cells infected with vTF7-3 recombinant vaccinia virus were transfected with pT7-GLT-1 (a; open symbols) or pT7-GAT-1 DNA (filled symbols) using plates containing 24 wells¹⁴. Transport was followed using 0.4 μ Ci of L-[³H]glutamate (60 Ci mmol⁻¹) in 200 μ l NaCl-containing transport medium (triangles) or medium with Na⁺ replaced by Li⁺ (○, ■)¹⁴. In b, transfection was with pT7-GLT-1. Reconstitution was done as described in Table 1 legend and for transport 5 μ Ci L-[³H]glutamate per transport reaction was used with increasing concentrations of unlabelled L-glutamate without (open circles) or with (closed circles) 2 μ M L-trans-pyrrolidine-2,4-dicarboxylate. Initial velocities (10-min time points) are plotted against glutamate concentration in a Lineweaver-Burk plot.

¶ To whom correspondence should be addressed.

Tissue-specific expression of the L-glutamate transporter was demonstrated by Northern blot analysis, which showed hybridization of a DNA sequence from the coding region to an 11-kb species from poly(A)⁺ RNA from rat brain, but not to messenger RNA from spleen, liver, heart, lung (Fig. 2a) or kidney (data not shown). The electrophoretic mobility of the hybridizing mRNA in the presence of methyl mercuric hydroxide²¹ was unaltered, suggesting that the low mobility was not due to incomplete denaturation of the mRNA.

Sequence analysis of the 4.6-kb insert indicates an open reading frame of 1,719 base pairs (Fig. 3a). Assignment of the first ATG as the translation initiation site is based on its resemblance to a consensus sequence²². The sequence codes for a protein of 573 amino acids and a relative molecular mass of ~64,000 (64K), in good agreement with the value of 63K of the purified and deglycosylated transporter⁷. The expressed protein was immunoprecipitated from lysates of HeLa cells labelled with [³⁵S]methionine by antibody raised against the glial L-glutamate transporter to yield a 65K band (Fig. 2b). This indicates that glycosylation is much less than the fully glycosylated transporter from rat brain, which runs as a 73K band^{7,9}. The cloned protein (Fig. 2b) and the purified native protein⁷ both run as relatively

broad bands and aggregate easily. A hydropathy plot²³ indicates that there are at least eight hydrophobic segments of 18–24 amino acids, long enough to span the membrane as an α -helix. The apparent absence of a signal sequence²⁴ suggests that the amino terminus is on the cytoplasmic side of the membrane. The even number of putative transmembrane α -helices suggests that the carboxy terminus is similarly located. Accordingly, the two potential glycosylation sites, which are located in a large loop between the putative helices 3 and 4, must be extracellular, whereas the only putative protein kinase A phosphorylation site and four of the six potential protein kinase C sites must be on the inside. A possible two-dimensional arrangement is depicted in Fig. 3b. There is another fairly long but less hydrophobic stretch between amino acids 346 and 369, but we did not assign this as a transmembrane α -helix because not only is it less hydrophobic but also the only potential protein kinase A phosphorylation site at (threonine position 385) and one of the potential protein kinase C sites at (Thr 370) would then be on

TABLE 1 Ion dependence and inhibitor sensitivity of L-[³H]glutamate transport in liposomes inlaid with proteins from HeLa cells transfected with GLT-1 cDNA

Ion dependence	Internal medium	
	Potassium phosphate	Tris-phosphate
External medium		
NaCl	5.8 ± 0.3	0.15 ± 0.04
LiCl	0.18 ± 0.04	0.06 ± 0.02
NaCl (no valinomycin)	2.6 ± 0.2	0.04 ± 0.02
NaSCN (no valinomycin)	5.2 ± 0.2	0.03 ± 0.01
Inhibitor sensitivity		
Control	7.0 ± 0.2	
+L-glutamate, 2 μ M	2.2 ± 0.1	
+L-glutamate, 5 μ M	1.2 ± 0.05	
+L-glutamate, 20 μ M	0.4 ± 0.03	
+L-glutamate, 100 μ M	<0.1	
+D-glutamate, 20 μ M	5.1 ± 0.2	
+D-glutamate, 100 μ M	3.7 ± 0.2	
+ γ -aminobutyrate, 20 μ M	7.3 ± 0.1	
+ γ -aminobutyrate, 100 μ M	6.3 ± 0.2	
+noradrenaline, 20 μ M	6.5 ± 0.1	
+noradrenaline, 100 μ M	6.1 ± 0.1	
+dopamine, 20 μ M	6.6 ± 0.1	
+dopamine, 100 μ M	6.2 ± 0.3	
+serotonin, 20 μ M	5.6 ± 0.1	
+serotonin, 20 μ M	5.6 ± 0.1	
+dihydrokainate, 20 μ M	0.92 ± 0.1	
+dihydrokainate, 100 μ M	0.2 ± 0.05	
+L-amino adipate, 20 μ M	2.0 ± 0.1	
+L-amino adipate 100 μ M	1.3 ± 0.1	

HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 units per ml penicillin, 100 μ g per ml streptomycin and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 and subsequent transfection with plasmid DNA was done as described¹⁴ using pT7-GLT-1 at 2.8 μ g per well (3-cm diameter). Cells from two wells were washed twice with 1 ml PBS then taken up in 20 μ l PBS. The cell suspension was mixed with liposomes (4 μ mol asolectin and 0.7 μ mol brain phospholipids) and 0.9% cholate in a final volume of 220 μ l and reconstituted using spin columns³⁰. The transport of L-[³H]glutamate in the proteoliposomes was then measured as described⁹ with valinomycin present at 2.5 μ M unless indicated. Transport was for 10 min, measured in triplicate. In this system the transport rates were linear for at least 20 min. Results are presented as mean $\pm$ s.d. (pmol min⁻¹ mg protein⁻¹).

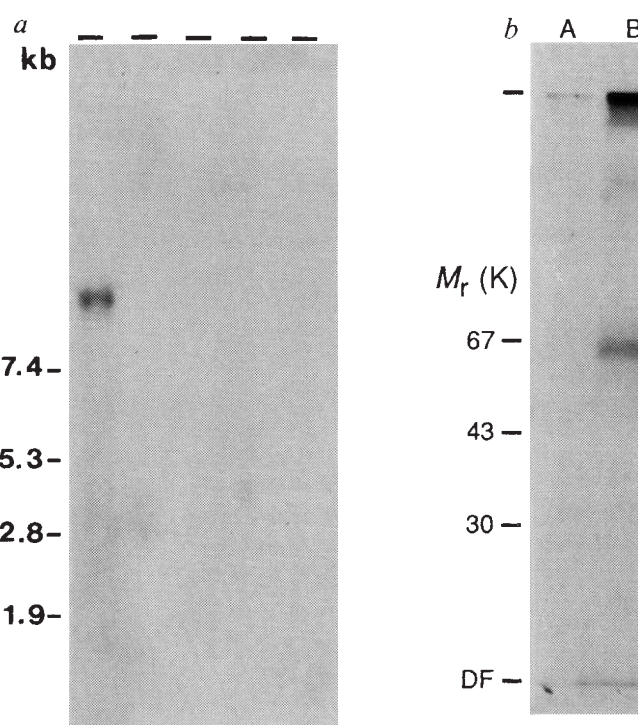


FIG. 2 *a*, Northern blot analysis of the cDNA clone, and *b*, immunoprecipitation of the GLT-1 protein by an antibody to the L-glutamate transporter from rat brain. In *a*, the cDNA probe hybridizes with rat brain mRNA, but not with mRNA from lung, liver, heart, spleen (from left to right in figure) or kidney (not shown). Each lane contained 5 μ g mRNA. The same results were found for 3 different RNA extractions. For western blot in *b*, immunoprecipitates of HeLa cell lysates were prepared using 10 μ l preimmune (lane A) or anti-glutamate transporter serum⁷ (lane B) with protein A Sepharose-CL-4B and the products analysed by autoradiography after SDS-PAGE¹⁴. The electrophoretic pattern of the cloned transporter is the same as that of the deglycosylated form of the transporter purified from rat brain and used to generate the antibody (see Fig. 5 in ref. 7). DF, dye front.

METHODS. mRNA was isolated from rats using oligo(dT)₂₅ magnetic particles (Dynabeads, from Dynal, Oslo) and separated on a 0.8% agarose gel with 6.7% formaldehyde, blotted onto a nylon membrane (Gene-Screen Plus; NEN) by capillary transfer, then hybridized to the cDNA probe corresponding to nucleotides 1,220–1,690 labelled with ³²P by random priming (T7 Quick-Prime kit; Pharmacia). Blots were washed for 2 $\times$ 10 min in 2 $\times$ SSC buffer at room temperature, 2 $\times$ 30 min in 0.1 $\times$ SSC, 0.5% SDS at 65 $^{\circ}$ C, then for 2 $\times$ 30 min in 0.1 $\times$ SSC at room temperature and autoradiographed for 1 week. Rehybridization with a chicken brain β -actin probe (Pst1 fragment) gave a single band at 2 kb³¹ in all lanes. For preparation of immunoprecipitates (*b*), HeLa cells were infected with vTF7-3 recombinant virus, transfected with pT7-GLT-1, labelled with [³⁵S]methionine (1,000 Ci mmol⁻¹)¹⁴ and then lysed.

1 ATGGCATCAACGAGGGTCCCAACATATGCCAAGCAGGTGAAGTGCATGACGAC 60
 1 M A S T E G A N N M P K Q V E V R M H D 20
 61 AGCCACCTCAGCTCCGAGGAGCCAAAGCAGCAAACTGGGATGCGATGCGACAAG 120
 21 S H L S S E E P K H R N L G M R M C D K 40
 121 CTGGGGAAGAACCTCTGCTCACTGACTGTGTGGTGTCTATCTGGGACAGTATGT 180
 41 L G K N L L L S L T V F G V I L G A V C 60
 181 GCGGGCTGCTTCGCTTGGCGGCTCCATCCACCTGATGTGGTGTATGATGCTTC 240
 61 G G L L R L A A P I H P D V V M L I A F 80
 241 CCGGGGACATCTCATGAGGATGCTGAAGATGCTCATCTCCCTCATCATTTCCAGT 300
 81 P G D I L M R M L K M L I L P L I I S S 100
 301 CTATCAGAGGCTGTGAGGCTGGATGCTAAAGCAGGCGCTAGGACGAGGCC 360
 101 L T T G L S G L D A K A S G R L G T R A 120
 361 ATGGTATTACATGTCACGACCATTCATGCGCTGTGTGGGGTCTCATCTGGTGTG 420
 121 M V Y Y M S T T I I A A V L G V I L V L 140
 421 GCCATCCACCTGGGAATCCCAACTCAAGAAGCAGCTGGGCTGGGAAGAAGACGAC 480
 141 A I H P G N P K L K K Q L G P G K K N D 160
 481 GAGGTGTCAGCTGGATGCTTCTGGATCTATTGAAGATCTCTCCGAGAACCTG 540
 161 E V S S L D A F L D L I R N L F P E N L 180
 541 GTACAGCCTGTTTCCACAGATTCAGACTGTGACAAGAAGTCTGGGACCTCCA 600
 181 V Q A C F Q Q I Q T V T K K V L V A P P 200
 601 TCGAGAGGCAATACAAACAGGAGCTCATCTCCCTGTTGAAGAGACATGAATGAG 660
 201 S E E A N T T K A V I S L L N E T M N E 220
 661 GCCCTGAAGAACTAAGATCGTTATCAAGAGGCGTGGATCAAGGACGGATGAAT 720
 221 A P E E T K I V I K K G L E F K D G M N 240
 721 GTCTAGGTCTGATGATCTTTATGCTTTCGCGATGCCATGGGAGATGGGTGA 780
 241 V L G L I G F F I A F G I A M G K M G V 260
 781 GCAGGCAAGCTGATGGTGGAGTCTTCAACATCTGAACAGATGTGATCAAGTATG 840
 261 A G Q A D G G V L Q H S E R D C H E V S 280
 841 GATCATGATGTGGTATTCGCGCTGGTATGCGCTGCTGATGTGGGAGATCATC 900
 281 D H D H V V F P A G I A C L I C G K I I 300
 901 GCCATCAAGGACTAGAGTGGTGTGAGGAGCTGGGATGATCATGATCAGTGTG 960
 301 A I K D L E V V A R Q L G M Y M I T V I 320
 961 GTGGCCTCATCATTCATGGGACATCTTCTCCCTGATTTACTTGTAGTGACGAGA 1020
 321 V G L I I H G G I F L P L I Y F V Y T R 340
 1021 AAAACCCATTCCTTTTGTGGGATTTTCAAGCTGGATCACTGCCCTGGGAAC 1080
 341 K N P F S F F A G I F Q A W I T A L G T 360
 1081 GCTTCCAGTGTGGAACCTTGGCTGTCACCTTCGCTGCTGAAGATATCAGGAT 1140
 361 A S S A G T L P V T F R C L E D N L G I 380
 1141 GACAAGCTGTGACGAGTTCGCTCCAGTGGAGCAACCATTAACATGGATGATACA 1200
 381 D K R V T R F V L P V G A T I N M D G T 400
 1201 GCCCTTACGAAGCGTGGCAGCATCTTATAGCCAGATGAATGGGTCATCTGGAT 1260
 401 A L Y E A V A C C I F A T G C A G A G T G A A T G G G T C A T C T G G A T 420
 1261 GGAGTGCAGATGACTGATGAGCTTACAGCACTCTGGCAGCATTTGGTGCAGCCAGT 1320
 421 G G Q I V T V S L T A T L A S I G A A S 440
 1321 ATCCAGCGCGGGTGTGACCATGCTCTCATCTTACAGCTGTGGGCTGGCGACA 1380
 441 I P S A G L V T M L L I L T A V G L P T 460
 1381 GAGGACATCAGTCTGCTGGTGGCAGTGGATGCTGCTGATAGAATGAGAATCTGGTC 1440
 461 E D I S L L V A V D W L L D R N R T S V 480
 1441 AATGTAGTGGGCGATCTTTTGGGCTGGGATGTCTACCTTTCCAAGTCCGAGCTG 1500
 481 N V V G D S F G A G I V Y H L S K S E L 500
 1501 GACACATTGACTCCCAACCAAGATGACGAGGACATCGAATGACCAAGACGAGTCC 1560
 501 D T I D S Q H R M H E D I E M T K T Q S 520
 1561 GTTATGACGACACGAAGAACCAAGGAACTCTAATCAGTGTGTCTATGCGGCA 1620
 521 V Y C D D T K N H R E S N S N A G C V Y A A 540
 1621 CACAACCTGTCTGATAGATGAGTGAAGTAACTGTGGCGGCAATGAAAGTCAAGCT 1680
 541 H N S V V I D E C K V T L A N G K S A 560
 1681 GACTGAGTGTGGAGGAAGAACCTTGAAGCTGAAATAATGACCCGATTCTCAGCCA 1740
 561 D C S V E E E P W K R E K * 573
 1741 ATTCTGAATAACTCCCGAGGTATCTTGTGTGAAAGATGCTTAAACAGGCTTCCTT 1800

the outside. Databank searches indicate that the transporter encoded by GLT-1 does not share significant overall homology with any known eukaryotic protein, including the Na⁺/glucose transporter and the growing superfamily of neurotransmitter transporters. This superfamily (for a review, see ref. 25) contains the transporters for the transmitters GABA, noradrenaline, serotonin, dopamine and glycine, as well as the choline moiety derived from acetylcholine; it also contains transporters for proline and betaine. The antibody used to isolate the clone GLT-1 specifically labels neuroglial cells⁷. The glial origin of the L-glutamate transporter encoded by GLT-1 was also confirmed by an experiment in which a 15-amino-acid peptide (amino acids 12–26: KQVEVRMHDHSLSSSE) was coupled to *N*-hydroxysuccinimide esters of agarose gel beads (Affi-Gel 15; BioRad). The immobilized peptide was then used to affinity-purify sequence-specific antibodies from IgG isolated from the L-glutamate transporter antiserum. This sequence-specific antibody fraction, like the total antibody preparation⁷, stained glial cells in different brain regions (data not shown). There is evidence for at least one more type of L-glutamate transporter of neuronal origin^{26,27} and for regional differences in transport pharmacology^{19,20}, so GLT-1 could be the first of a new family of transporters.

Discrete regions of GLT-1 show significant homology with the proton-coupled L-glutamate transporter glt-P of *Escherichia coli*²⁸, including a 40% identity in the stretch of amino-acid residues from 406 to 514. Particularly interesting is the identity

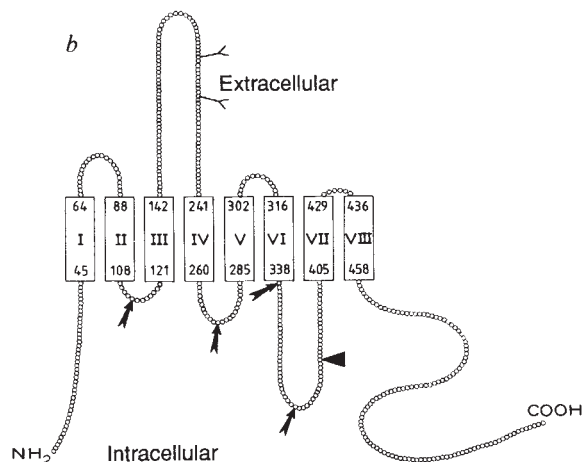


FIG. 3 *a*, Nucleotide and deduced amino-acid sequence of pT7-GLT-1. The first nucleotide and amino acid residue of the translation start site are designated as position 1. Only part of the 3' untranslated region is shown. The EMBL data library accession number is X67857. Putative transmembrane domains as predicted by Kyte-Doolittle analysis are underlined. *b*, Schematic representation of the L-glutamate transporter showing its proposed orientation in the plasma membrane. Individual amino-acid residues are shown as circles and putative transmembrane segments as rectangles. One putative protein kinase A and four putative protein kinase C phosphorylation sites are indicated by an arrowhead and arrows, respectively. The remaining two potential protein kinase C sites in the putative extracellular loop between helices 3 and 4 are not shown. The two putative glycosylation sites are also located on this loop and are indicated as branched lines.

METHODS. A rat brain λ zap library⁸ was immunoscreened³² using affinity-purified antibody directed against the glial L-glutamate transporter from rat brain⁷. Positive clones were rescued according to the protocol of Stratagene and characterized with regard to insert size, restriction map and expression^{12–14}. DNA sequencing³³ of clones identified several that contained open reading frames with significant homology with glt-P (ref. 28), the proton-coupled L-glutamate transporter of *E. coli*. Immunoblots with antibody from lysates of *E. coli* harbouring bBluescript containing such an insert yielded a fusion protein of *M*_r 25K, which is significantly shorter than the purified transporter from rat brain⁹. The main part of its coding region the 470-bp *Pst*I fragment, was used to rescreen another λ zap library^{10,11}. Several of the positive clones were rescued and expressed in the vaccinia/T7 system^{12–14}. One of these that directed expression in this system was pT7-GLT-1. DNA sequencing was done from both strands using a Sequenase version 2.0 kit (US Biochem) by 'walking' from both ends. Ambiguities were solved by repeating sequencing reactions using dITP rather than dGTP.

of seven amino acids (407–413; AAIFIAQ) between the *E. coli* and rat brain L-glutamate transporters. As the coupling ions are different in these transporters, the domain containing this sequence may be involved in binding glutamate.

Glutamate has been implicated in mental processes such as learning and memory, in brain damage following ischaemia or hypoglycaemia, as well as in neurological disorders like Alzheimer's and Parkinson's disease, Huntington's chorea and epilepsy and amyotrophic lateral sclerosis. The last condition may be associated with a specific defect in glutamate transport²⁹. Our cDNA clone will be useful for analysing defects accompanying this disease and others, in addition to contributing to our understanding of the transport process itself. □

Received 21 July; accepted 20 October 1992.

- Iversen, L. L. *Br. med. Bull.* **29**, 130–136 (1973).
- Bennett, J. P. Jr, Mulder, A. H. & Snyder, S. M. *Life Sci.* **15**, 1045–1056 (1974).
- Balcar, V. J. & Johnston, G. A. R. *J. Neurobiol.* **3**, 295–301 (1972).
- Kanner, B. I. & Schuldiner, S. *CRC Crit. Rev. Biochem.* **22**, 1–38 (1987).
- Nicolls, D. & Attwell, D. *Trends Pharmac. Sci.* **11**, 462–468 (1990).
- McBean, G. J. & Roberts, P. J. *J. Neurochem.* **44**, 247–254 (1985).
- Danbolt, N. C., Storm-Mathisen, J. & Kanner, B. I. *Neuroscience* **51**, 295–310 (1992).
- Keinanen, K. *et al. Science* **249**, 556–560 (1990).
- Danbolt, N. C., Pines, G. & Kanner, B. I. *Biochemistry* **29**, 6734–6740 (1990).
- Guastella, J. *et al. Science* **249**, 1303–1306 (1990).
- Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A. & Davidson, N. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3391–3395 (1990).
- Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8122–8126 (1986).
- Blakely, R. D., Clark, J. A., Rudnick, G. & Amara, S. G. *Analyt. Biochem.* **194**, 302–308 (1991).
- Keynan, S., Suh, Y.-J., Kanner, B. I. & Rudnick, G. *Biochemistry* **31**, 1974–1979 (1992).
- Kanner, B. I. & Sharon, I. *Biochemistry* **17**, 3949–3953 (1978).
- Kanner, B. I. & Bendahan, A. *Biochemistry* **21**, 6327–6330 (1982).
- Pines, G. & Kanner, B. I. *Biochemistry* **29**, 11209–11214 (1990).
- Bridges, R. J., Stanley, M. S., Anderson, M. W., Cotman, C. W. & Chamberlin, A. R. *J. med. Chem.* **34**, 717–725 (1991).
- Ferkany, J. & Coyle, J. T. *J. Neurosci. Res.* **16**, 491–503 (1986).
- Fletcher, E. & Johnston, G. A. R. *J. Neurochem.* **57**, 911–914 (1991).
- Perbal, B. *A Practical Guide to Molecular Cloning* 2nd edn (Wiley, New York, 1988).
- Kozak, M. *Nucleic Acid Res.* **15**, 8125–8148 (1987).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- von Heijne, G. *Eur. J. Biochem.* **133**, 17–21 (1983).
- Uhl, G. R. *Trends Neurosci.* **15**, 265–268 (1992).
- Text, T. & Storm-Mathisen, J. *Neuroscience* **11**, 79–100 (1984).
- Wilkin, G. P., Garthwaite, J. & Balázs, R. *Brain Res.* **244**, 69–80 (1982).
- Tolner, B., Poolman, B., Wallace, B. & Konings, W. N. *J. Bact.* **174**, 2391–2393 (1992).
- Rothstein, J. D., Martin, L. J. & Kuncel, R. W. *New Engl. J. Med.* **326**, 1464–1468 (1992).
- Radian, R. & Kanner, B. I. *J. Biol. Chem.* **260**, 11859–11865 (1985).
- Cleveland, D. W. *et al. Cell* **20**, 95–105 (1980).
- Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning* (eds Ford, N., Natan, C. & Ferguson, M.) 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).

ACKNOWLEDGEMENTS. We thank P. Seeburg for the $\lambda_{\text{gt}10}$ library from rat brain; and S. Pantanowitz, H. Johnsen, E. Disser, E. Hoff and S. Keynan for their help. Supported by the Basic Research Foundation administered by the Israel Academy of Sciences and Humanities (B.I.K.); by Anders Jahres Fond til Videnskaps Fremme and Rakel and Otto K. Bruuns Legat, Stefi og Lars Fylkesakers Vitenskapelige Stiftelse, and Norsk-Israelsk Forskningsfond (N.C.D., J.S.M.); and the Norwegian Cancer Society and the Norwegian Research Council for Science and the Humanities (E.S. and M.B.).

Primary structure and functional characterization of a high-affinity glutamate transporter

Yoshikatsu Kanai & Matthias A. Hediger*

Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115, and Department of Biological and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

GLUTAMATE transport across plasma membranes of neurons, glial cells and epithelial cells of the small intestine and kidney proceeds by high- and low-affinity transport systems^{1–5}. High-affinity (K_m 2–50 μ M) transport systems have been described^{1,6,7} that are dependent on Na^+ but not Cl^- ions and have a preference for L-glutamate and D- and L-aspartate. In neurons high-affinity

glutamate transporters are essential for terminating the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft^{6,7}. We have isolated a complementary DNA encoding an electrogenic Na^+ - but not Cl^- -dependent high-affinity glutamate transporter (named EAAC1) from rabbit small intestine by expression in *Xenopus* oocytes. We find EAAC1 transcripts in specific neuronal structures in the central nervous system as well as in the small intestine, kidney, liver and heart. The function and pharmacology of the expressed protein are characteristic of the high-affinity glutamate transporter already identified in neuronal tissues. The abnormal glutamate transport that is associated with certain neurodegenerative diseases⁸ and which occurs during ischaemia and anoxia⁷ could be due to abnormalities in the function of this protein.

To isolate a cDNA encoding a high-affinity glutamate transporter we used an expression cloning approach similar to that used to clone the Na^+ /glucose cotransporter⁹. This resulted in the isolation of a 3,442-base-pair (bp) cDNA. The nucleotide and deduced amino-acid sequences are shown in Fig. 1. We identified an open reading frame coding for a 524-amino-acid protein, which we named EAAC1 (excitatory amino-acid carrier 1). The protein has a predicted relative molecular mass of 57,000. The algorithm of Eisenberg¹⁰ predicts a topological model for the transporter with ten potential membrane-spanning regions (Fig. 1). Because of the presence of a large hydrophobic stretch near the C terminus (residues 357 to 444), alternative models with a different number of membrane-spanning regions are conceivable. The N-terminal part of EAAC1 is hydrophilic and lacks a signal peptide. A motif-like cluster of serine residues is indicated in the figure (residues 331–334). Similar clusters have been identified in the ligand-binding sites of receptors for acetylcholine and biogenic amines¹¹.

In high-stringency northern blots (Fig. 2a), prominent RNA bands of 3.5 kilobases (kb) were evident in samples from small intestine, kidney and brain, and faint bands of identical size were detected from liver and heart. There was a second band at 2.5 kb from some tissues such as in kidney and liver. The size of these two transcripts is consistent with the presence of polyadenylation sites at positions 2,487 and 3,407 (Fig. 1). In kidney the larger band is predominant in the superficial cortex, whereas the smaller band is more evident in the remaining cortex and the outer medulla, suggesting that different polyadenylation sites are used along renal tubules. The expression of EAAC1 in brain was further investigated using amplification by polymerase chain reaction (PCR) of RNA from cerebral cortex, cerebellum and hippocampus, with primers corresponding to the EAAC1 cDNA sequence. As shown in Fig. 2b, PCR amplification gave the same size of amplification products for cerebral cortex, cerebellum, hippocampus, jejunum and EAAC1 cDNA. Moreover, digestion of PCR products from cerebral cortex by the restriction enzymes *SalI*, *XbaI* or *NcoI*, which target internal cleavage sites on EAAC1 cDNA, gave a cleavage pattern identical to that from both jejunum and EAAC1 cDNA (data not shown), so confirming that the EAAC1 sequence is expressed in brain.

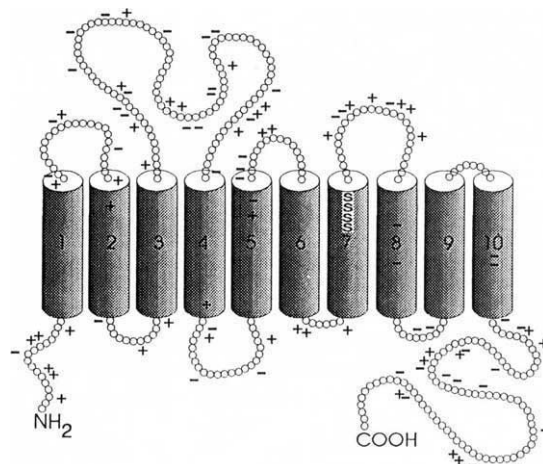
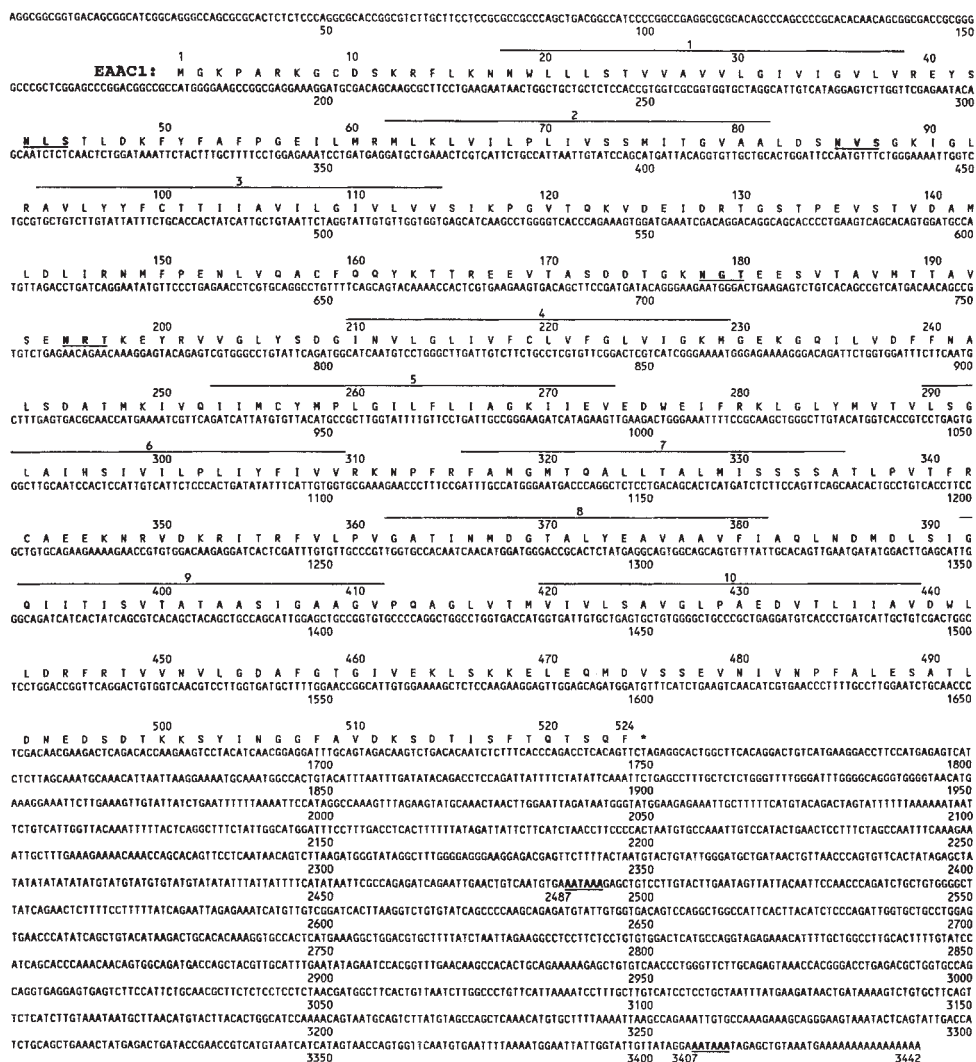
In situ hybridization (Fig. 3) using frozen sections showed that EAAC1 mRNA is abundantly present in epithelial cells of the small intestine (Fig. 3e and f) and in the hippocampal formation, specifically in the pyramidal layer from region CA1 to CA4, and the granular layer of the dentate gyrus (Fig. 3a). In cerebellum the EAAC1 mRNA is highly expressed in the granule cell layer (Fig. 3b). In the cerebral cortex, EAAC1 mRNA was identified in cells of layer II to VI (Fig. 3c and d). The most likely explanation for the EAAC1 signals in these layers is that EAAC1 is specifically expressed in neurons. Selective expression in neurons would suggest that there is a separate transport system in glial cells, although, to our knowledge, structural and functional distinctions between high-affinity glutamate transporters in neurons and in glial cells have not been found.

* To whom correspondence should be addressed at Brigham and Women's Hospital.

When expressed in *Xenopus laevis* oocytes, EAAC1, induces ^{14}C -glutamate uptake to 1,300-fold above that of water-injected controls (Fig. 4a). The uptake is totally Na^+ -dependent but is not Cl^- -dependent (Fig. 4a, b). EAAC1-mediated transport was electrogenic (Fig. 4c). Bath-applied L-glutamate and D- or L-aspartate induced a large inward current in oocytes injected with EAAC1 complementary RNA (Fig. 4c), but there was no significant current in water-injected oocytes (data not shown). The current was completely Na^+ -dependent, consistent with the ^{14}C -L-glutamate uptake studies. The dependence on concentra-

tion of the currents indicated that transport is saturable (Fig. 4d), with K_m values for L-glutamate, L-aspartate and D-aspartate of 12.2 ± 1.2 , 6.5 ± 0.7 and $7.5 \pm 0.7 \mu\text{M}$ (mean $\pm$ s.e.m., $n = 3$), respectively. These values are indicative of high-affinity transport^{1,7,12}. In accordance with the pharmacology of high-affinity glutamate transport¹³, the glutamate receptor ligands NMDA (*N*-methyl-D-aspartate), quisqualate and kainate evoked either no current or a small current (quisqualate). L-Homocysteate is a substrate of low-affinity but not of high-affinity glutamate transport¹⁴. Consistent with this, the current induced by L-

FIG. 1 Top, Nucleotide and deduced amino-acid sequence of EAAC1. The AUG initiation codon at position 177 matches the Kozak consensus initiation sequence [GCC(A/G)CCAUGG]³⁸. Putative transmembrane domains predicted by Eisenberg's algorithm¹⁰ are marked by lines 1–10. Potential *N*-linked glycosylation sites (Asn 43, 85, 178 and 195) and polyadenylation sequences (AATAAA) at position 2,487 and at 3,407 are underlined. Potential PKC-dependent phosphorylation sites (S/T-X-K/R; one-letter amino-acid code)³⁹ are located at residues 11, 87, 115, 121, 164, 175, 247, 340 and 466. Bottom, Membrane model of EAAC1. Putative transmembrane regions are depicted as cylinders. Charged amino acids (Arg, Lys, Glu, Asp) are indicated. The motif-like cluster of serine residues is indicated in membrane-spanning region 7 (residues 331–334). METHODS. RNA was extracted from jejunum mucosal scrapes of female New Zealand White rabbits by the guanidinium isothiocyanate method using caesium trifluoroacetate (Pharmacia). Poly(A)⁺ RNA was isolated and injected into collagenase-treated and manually defolliculated *Xenopus* oocytes as described⁴⁰. This induced significant Na^+ -dependent L-glutamate uptake (13-fold above that of water-injected controls). Size fractionation of rabbit jejunum poly(A)⁺ RNA using preparative gel electrophoresis⁴¹ and injection of fractions into oocytes showed peak stimulation of glutamate uptake by RNA in the size range 2.4–4.4 kb. A directional cDNA library was constructed from this size range using the SuperScript Plasmid System (GibcoBRL, MD). cDNA was ligated into the *Not*I and *Sal*I restriction enzyme digestion sites of the expression vector pSPORT 1 (GibcoBRL) and electroporated into ElectroMax DH10B cells (GibcoBRL). Plasmid DNA was transcribed *in vitro* from pools of 300–400 clones and the resulting cRNA injected into oocytes^{9,42}. A pool was identified that increased the uptake of glutamate 12-fold more than water-injected controls. This pool was sequentially subdivided and analysed until a single clone (EAAC1) was identified. Uptake experiments were done with 6–8 oocytes 3 d after injection. Incubation was for 1 h in 750 μl standard uptake medium (100 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM HEPES, 5 mM Tris, pH 7.4) and 50 μM ^{14}C -L-glutamate. EAAC1 cDNA or cDNA fragments produced by internal restriction sites were subcloned into pBluescript II (Stratagene) and were completely sequenced in both directions using the Sequenase 2.0 DNA sequencing kit (USB, OH); synthetic oligonucleotide primers were used to complete sequencing.



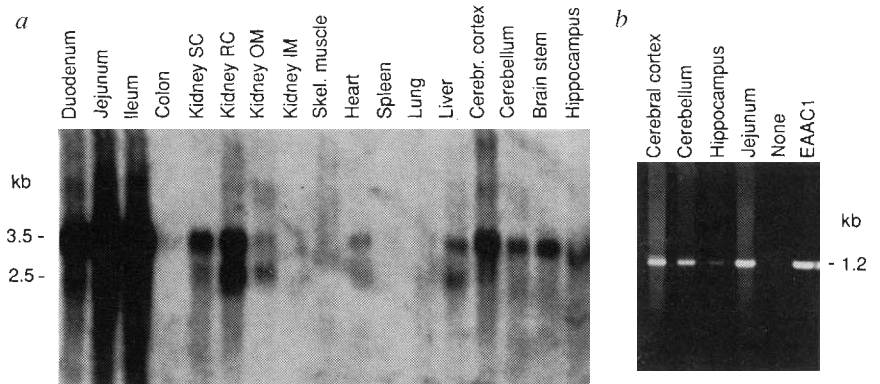
homocysteate is small compared with that evoked by L-glutamate (Fig. 4c). Likewise, L-homocysteate does not significantly inhibit uptake of ^{14}C -L-glutamate and L-aspartate (data not shown). Of the other amino acids tested (at 100 μM), L-glutamine and L-asparagine induce a small inward current (5–6% of the current evoked by L-glutamate), whereas L-alanine, L-leucine, L-lysine, L-arginine, L-cystine, β -alanine, taurine, L-proline and γ -aminobutyric acid (GABA) do not evoke significant currents.

The stereospecificity of EAAC1-mediated glutamate and aspartate transport (Fig. 4c) was confirmed by inhibition experiments in which we measured the uptake of ^{14}C -L-glutamate or

^{14}C -L-aspartate (50 μM) in the presence and absence of an excess of unlabelled glutamate or aspartate stereoisomers (1 mM). The uptake of ^{14}C -L-glutamate was effectively inhibited by L-aspartate and D-aspartate (93.6% and 93.1% inhibition respectively), but not by D-glutamate (14.4% inhibition); uptake of ^{14}C -L-aspartate was prevented by D-aspartate and L-glutamate (82.2% and 79.4% inhibition respectively), whereas D-glutamate was not an efficient inhibitor (36.2% inhibition). We conclude that the kinetics and stereospecificity of EAAC1 match those of high-affinity Na^+ -dependent glutamate transport systems already described for neurons, glial cells and epithelial cells^{1–3,12,13}.

FIG. 2 a, High-stringency northern analysis of RNA from rabbit tissues probed with ^{32}P -labelled EAAC1 cDNA. A strong 3.5-kb band was detected in duodenum, jejunum, ileum, kidney superficial cortex (SC) and remaining cortex (RC) and brain samples, particularly cerebral cortex. A weak band of identical size was present in liver and heart. An additional 2.5-kb band of varying intensity was detected in all positive tissues except brain. The band in hippocampus was slightly smaller. This is probably because more total RNA was loaded on the gel than poly(A)⁺ RNA (10 μg of total RNA for hippocampus and 3 μg of poly(A)⁺ RNA for all other tissues). **b**, Detection of EAAC1 mRNA expression by PCR. PCR amplification of first strand cDNA from cerebral cortex, cerebellum and hippocampus shows amplification products of the same size as those detected in jejunum and obtained from EAAC1 cDNA. For the reverse transcription reactions, the same amounts of poly(A)⁺ RNA were used for cerebral cortex, cerebellum and jejunum. Total RNA was used for hippocampus.

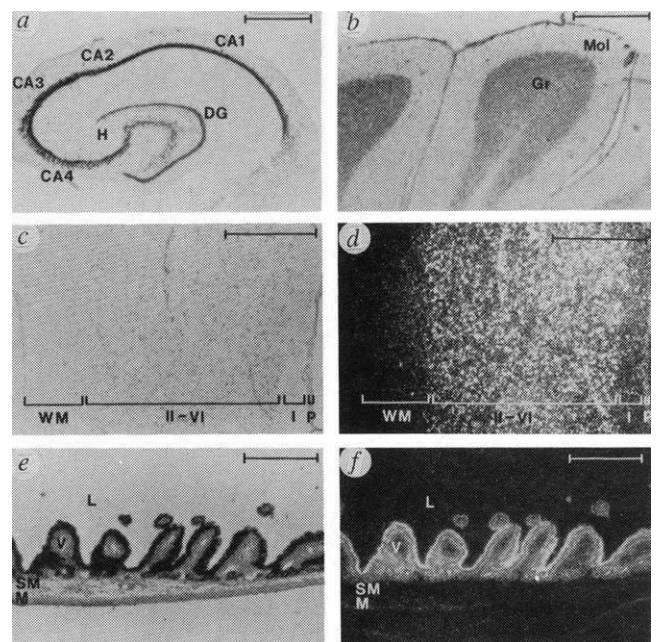
METHODS. **a**, Poly(A)⁺ RNA (3 μg) or total RNA (10 μg ; hippocampus) was separated on a 1% formaldehyde agarose gel, and blotted onto a nitrocellulose filter (S&S). The EAAC1 insert was excised from pSPORT1 and labelled with ^{32}P using ^{32}P -QuickPrime kit (Pharmacia). Hybridization was for 16 h at 42 °C (50% formamide). The filter was washed in 0.1 $\times$ SSC/0.1% SDS at 65 °C. **b**, For PCR analysis of EAAC1 expression, first strand cDNA was synthesized from 0.5 μg poly(A)⁺ RNA from cerebral cortex, cerebellum and



jejunum and 2.5 μg total RNA from hippocampus using oligo(dT)_{12–18} as a primer. Subsequently, a sense primer (5'-GACAGATTCTGGTGGATTCTTC-3') and an antisense primer (5'-ATACTAGTCTGTACATGAAAAG-3'), corresponding to nucleotides 874–896 and 2,062–2,084, respectively, were used to amplify 1% of the products from the reverse transcription reaction or 0.5 μg EAAC1 cDNA subcloned in pBluescript II for 30 cycles (10 s at 92 °C, 1 min at 55 °C, 2 min at 72 °C). PCR products were separated by electrophoresis through a 1% agarose gel and stained with ethidium bromide.

FIG. 3 Localization of the EAAC1 message in brain and intestinal tissues by *in situ* hybridization. **a**, Hippocampal formation: The EAAC1 antisense cRNA probe strongly hybridized to the pyramidal layer (from CA1 to CA4) and granular layer of the dentate gyrus (DG). The figure shows hybridization in the pyramidal layer extending into the hilus (H) and subiculum. **b**, Folia of cerebellum: dense hybridization was observed in the granule cell layer (Gr), although to a lesser extent compared with that of the pyramidal layer (note that **b** is a 10-day exposure and **a** is a 5-day exposure). Mol, molecular layer. **c** and **d**, Cerebral cortex: **c** is a bright-field photograph of a counter-stained slide and **d** is a dark-field photograph of the same area. Grains were accumulated on cells in layer II–VI and not detected in deep white matter (WM). P, pia mater. **e** and **f**, Jejunum: **e** is a bright-field photograph of the counterstained slide and **f** is a dark-field of the same area. Strong hybridization was detected on epithelial cells and grains were not detected in submucosal layer (SM) and muscle layer (M). V, villi; L, lumen. Control experiments using sense EAAC1 cRNA as a probe were found to have only background levels of hybridization (data not shown). Scale bars in **a**, **c** and **d**, 1 mm; **b**, **e** and **f**, 0.5 mm.

METHODS. *In situ* hybridization of rabbit jejunum and brain tissues (hippocampus, cerebellum and cerebral cortex) was done as described with some modifications⁴³. After perfusion fixation with 4% paraformaldehyde, hippocampus, cerebellum and cerebral cortex were removed and postfixed in 4% paraformaldehyde. Jejunum was taken under deep anaesthesia and fixed by immersing in 4% paraformaldehyde. Two serial cryosections (9- μm for jejunum and 18- μm for brain tissues) were prepared for *in situ* hybridization. ^{35}S -labelled sense and antisense RNA probes were synthesized from a subclone (646–2783 *StuI*–*StuI* EAAC1 fragment ligated into the pBluescript II *EcoRV* site) after linearization with *EcoRI* or *HindIII*, using T7 or T3 RNA polymerase, respectively. RNA probes were degraded by partial hydrolysis for 40 min to form probes of ~100 nucleotides. After being treated with proteinase K (at 2 $\mu\text{g ml}^{-1}$ for 7.5 min) and acetylated with acetic anhydride, cryosections were hybridized with probes at 50 °C overnight in the hybridization solution (50% formamide). Sections were washed in 5 $\times$ SSC for 30 min at 50 °C, in 50% formamide and 2 $\times$ SSC for 20 min at 50 °C and then in



0.4 M NaCl for 20 min at 37 °C. The sections were treated with RNase A (40 $\mu\text{g ml}^{-1}$) and RNase T1 (2 $\mu\text{g ml}^{-1}$) at 37 °C for 30 min and washed in 0.1 $\times$ SSC at 37 °C for 15 min. Air-dried slides were dipped into Kodak NTB2 emulsion and developed 5–10 days later. Some brain and jejunum slides were counterstained with cresylviolet and haematoxylin–eosin, respectively.

The pharmacological properties of EAAC1-mediated transport were further evaluated using inhibitors of L-glutamate uptake that had previously been characterized using synaptosomes^{12,15}. DL-Threo- β -hydroxyaspartate (THA), a strong inhibitor of L-glutamate uptake in synaptosomes, induced an inward current in oocytes injected with EAAC1 cRNA ($K_m = 6.9 \pm 0.6 \mu\text{M}$, mean $\pm$ s.e.m.; $n = 3$), which is consistent with results from salamander retinal glial cells¹⁶. L- α -Aminoadipate and dihydrokainate, which are less effective as inhibitors, also induced inward currents, with K_m values of $201 \pm 6 \mu\text{M}$ and $>1 \text{ mM}$, respectively ($n = 3$). Consistent with this, the current evoked by $20 \mu\text{M}$ L-glutamate was inhibited by THA (concentration giving 50% inhibition, $\text{IC}_{50} = 7.1 \pm 0.4 \mu\text{M}$), amino adipate ($\text{IC}_{50} = 165 \pm 21 \mu\text{M}$) and dihydrokainate ($10.0 \pm 1.4\%$ inhibition at 1 mM ; $n = 3$). This order of inhibition (THA $>$ amino adipate $>$ dihydrokainate) of EAAC1-mediated transport is the same as for the glutamate transporter subtype that is a major component in cerebellar synaptosomes and also participates in brain stem and forebrain synaptosomal glutamate transport^{12,15}.

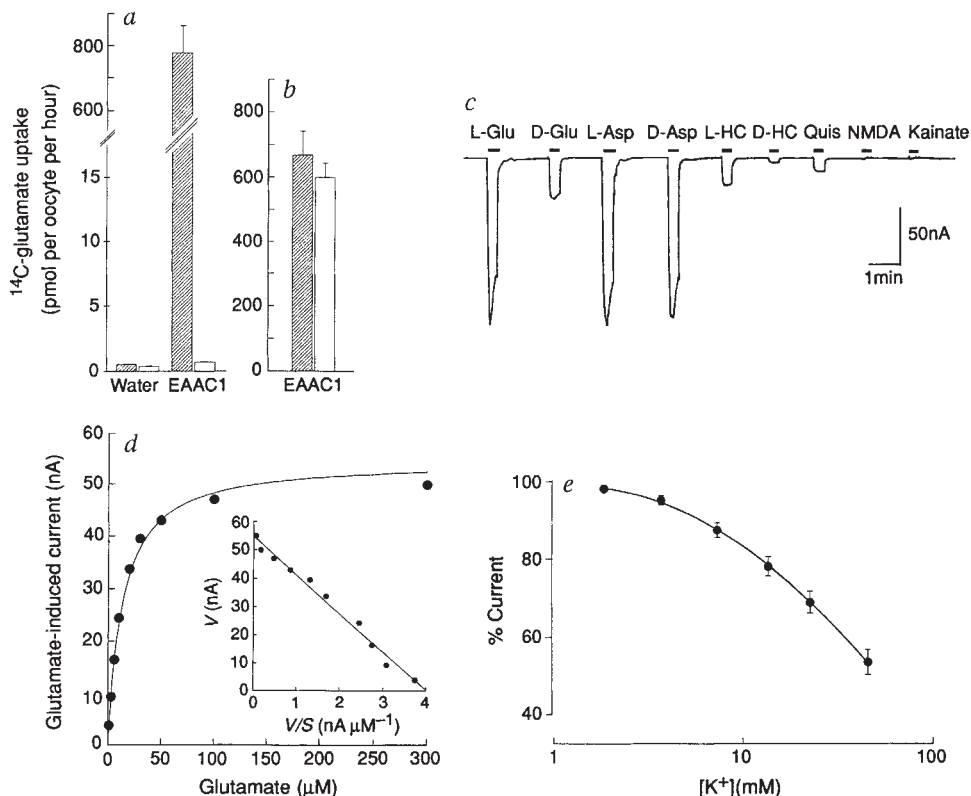
The role of K^+ in high-affinity glutamate transport is con-

troversial^{2,16-19}. The proposed K^+ countertransport model^{7,16} was tested by monitoring the dependence of L-glutamate-induced current on extracellular K^+ concentration. This experiment used voltage-clamp conditions to ensure that changing the extracellular K^+ concentration did not affect glutamate transport through alteration of the membrane potential. Our experiments show that L-glutamate-induced current was significantly suppressed at higher extracellular K^+ concentration (Fig. 4e), which is consistent with the K^+ -countertransport model^{7,16}.

A search of protein sequence databases (June 1992) revealed weak but significant sequence similarities between EAAC1 and prokaryotic transporters. We found that EAAC1 has 30% identity with the *Escherichia coli* GltP proton/glutamate-aspartate symporter²⁰ and 27% identity with the DctA dicarboxylate transporter from *Rhizobium meliloti*²¹. There was no significant homology between EAAC1 and the *E. coli* Na^+ /glutamate transporter GltS²², the Na^+ -glucose transporter and related transporters^{9,23} or to Na^+ - and Cl^- -dependent transporters of GABA²⁴, noradrenaline²⁵, dopamine^{26,27}, serotonin^{28,29}, L-glycine³⁰ and L-proline³¹. The similarities of the glt-P, DctA and EAAC1 sequences, together with the absence of significant sequence

FIG. 4 a, Uptake of ^{14}C -labelled L-glutamate into *Xenopus* oocytes. Oocytes were injected with water or *in vitro*-transcribed EAAC1 cRNA. Each column represents the mean $\pm$ s.e.m. ($n = 6-8$ oocytes). Vertical bars give the standard error. Hatched columns indicate uptake values in the presence of Na^+ (100 mM); open columns show uptake in Na^+ -free medium in which Na^+ has been replaced by choline. Uptake into EAAC1 cRNA-injected oocytes demonstrated complete Na^+ -dependence. b, Effects of Cl^- on EAAC1-mediated L-glutamate uptake. Hatched column indicates ^{14}C -L-glutamate uptake into EAAC1 cRNA-injected oocytes measured in the standard uptake solution ($[\text{Cl}^-] = 106 \text{ mM}$). The open column shows uptake in the absence of Cl^- (Cl^- was replaced by acetate). The uptake did not show significant dependence on Cl^- concentration in the medium. c, Current responses of *Xenopus* oocytes injected with EAAC1 cRNA to bath-applied $100 \mu\text{M}$ substrates. Membrane current was measured in standard bath solution (ND 96) at a holding potential of -60 mV . Substrates were applied at the points indicated by overbars. Inward current is directed downwards. L-Glutamate, L-aspartate and D-aspartate evoked almost the same amplitude of inward current, whereas currents induced by D-glutamate and L- or D-homocysteate (HC) were small compared with L-glutamate. The glutamate receptor ligands NMDA and kainate did not induce current. Quisqualate (Quis), however, evoked a small but significant inward current. d, Representative concentration dependence of EAAC1-mediated L-glutamate transport. L-glutamate-evoked currents of the EAAC1 cRNA-injected oocyte were measured at -60 mV at varied L-glutamate concentration. EAAC1-mediated L-glutamate transport was saturable and the K_m value calculated from the Eadie-Hofstee plot (inset) is $12.2 \pm 1.2 \mu\text{M}$ (mean $\pm$ s.e.m.; $n = 3$). e, Inhibition of L-glutamate-induced current by external K^+ . Currents evoked by $50 \mu\text{M}$ L-glutamate were measured at various K^+ concentrations (bath medium, 0 mM – 50 mM) at a holding potential of -30 mV and normalized to the current measured on the same oocyte (the value of the current at 0 mM K^+ is 100%). Each point represents the mean $\pm$ s.e.m. from 6 oocytes. Abscissa indicates external K^+ concentration on a logarithmic scale. Inhibition of current was greater at higher K^+ concentration.

METHODS. Oocytes were treated and injected with EAAC1 cRNA (25 ng per oocyte) as described in Fig. 1a. Uptake of ^{14}C -glutamate ($50 \mu\text{M}$) was measured 3 days after injection in standard uptake medium (Fig. 1) and in



Na^+ -free medium in which NaCl was replaced by choline- Cl . In b, Cl^- in the uptake medium is replaced by acetate. In c, electrophysiological measurements were made 3–4 d after injection using the conventional two-microelectrode voltage-clamp method (Axoclamp-2A, Axon Instruments). The standard bath solution (ND 96) contained 96 mM NaCl , 2 mM KCl , 1.8 mM CaCl_2 , 1.0 mM MgCl_2 and 5 mM HEPES , pH 7.4. The recording chamber was perfused with bath solution and substrates were applied at the bars. In d, electrophysiological measurements were made at -60 mV . Current amplitudes measured at different L-glutamate concentrations (1 – $300 \mu\text{M}$) were plotted and the curves fitted using the Michaelis-Menten equation; the K_m was calculated using the Eadie-Hofstee equation. e, L-Glutamate-induced current was measured as in c, but over a range of K^+ concentrations (0 – 50 mM). Consecutive current measurements were made on single oocytes after changing to bath medium containing the appropriate K^+ concentration. After stabilization of the membrane current, $50 \mu\text{M}$ of L-glutamate was applied. Bath medium contained $x \text{ mM KCl}$ (where $x = 0$ – 50), $50 - x \text{ mM choline-Cl}$, 50 mM NaCl , 1.8 mM CaCl_2 , 1.0 mM MgCl_2 , 10 mM BaCl_2 , 5 mM HEPES , pH 7.4.

homology with Na^+ and Cl^- -dependent neurotransmitter transporters or to any other published sequence, establish that EAAC1, GltP and DctA define a new protein superfamily.

Although EAAC1 cDNA was isolated from small intestine, our results demonstrate that it is expressed in brain. The localization of the EAAC1 mRNA in general matches the location of cell bodies of glutamatergic neurons³². This suggests that EAAC1 is specifically expressed in neurons and that it may function as a presynaptic glutamate uptake carrier. Under normal conditions, high-affinity glutamate transport prevents the extracellular glutamate concentration from reaching neurotoxic levels⁷, so its chronic suppression is implicated in neuronal degeneration^{33,34}. For example in amyotrophic lateral sclerosis, a neurodegenerative disease, there is a reduction of high-affinity glutamate transport which may be involved in the pathophysiology⁸. EAAC1 in the presynaptic membrane would be ideally placed to remove glutamate from synaptic clefts and any defect in its function would probably severely affect the nervous system.

In some pathological conditions such as brain ischaemia and anoxia, the extracellular K^+ concentration is raised, the Na^+ concentration reduced and the membrane depolarized^{7,35,36}. Under these conditions, which are unfavourable for glutamate uptake, EAAC1 may operate in the reversed direction. Reversed glutamate transport has been demonstrated using salamander retinal glial cells³⁷ and may be a major cause of the pathological rise in extracellular glutamate that leads to neuronal death⁷.

Once the cellular and subcellular localization of the EAAC1 protein has been established and its kinetics analysed, it will be possible to define its role in synaptic transmission and to address issues that are related to the regulation of synaptic and extracellular glutamate concentration. □

The glial cell glutamate uptake carrier countertransports pH-changing anions

Muriel Bouvier, Marek Szatkowski*,
Alessandra Amato & David Attwell

Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK

* Department of Physiology and Biophysics, St Mary's Hospital Medical School, Imperial College of Science, Technology & Medicine, Norfolk Place, London W2 1PG, UK

UPTAKE into glial cells helps to terminate glutamate's neurotransmitter action and to keep its extracellular concentration, $[\text{Glu}]_o$, below neurotoxic levels. The accumulative power of the uptake carrier stems from its transport of inorganic ions such as sodium (into the cell) and potassium (out of the cell)¹⁻³. There is controversy over whether the carrier also transports a proton (or pH-changing anion)^{4,5}. Here we show that the carrier generates an alkalization outside and an acidification inside glial cells, and transports anions out of the cells, suggesting that there is a carrier cycle in which two Na^+ accompany each glutamate anion into the cell, while one K^+ and one OH^- (or HCO_3^-) are transported out. This stoichiometry predicts a minimum $[\text{Glu}]_o$ of $0.6 \mu\text{M}$ normally (tonically activating presynaptic autoreceptors and post-synaptic NMDA receptors), and $370 \mu\text{M}$ during brain anoxia (high enough to kill neurons). Transport of $\text{OH}^-/\text{HCO}_3^-$ on the uptake carrier generates significant pH changes, and may provide a mechanism for neuron-glial interaction.

Glutamate uptake was monitored electrically⁶ in salamander retinal glia⁷, which have uptake like that in mammalian glia⁸ and neurons¹ but have no glutamate-gated ion channels^{3,7}. Changes of extracellular pH (pH_o) were monitored just outside the cell while controlling the membrane potential by whole-cell clamping. Activating uptake, by hyperpolarizing in the presence of external glutamate⁷, led to the pH_o going alkaline (Fig. 1a). Omitting external glutamate (Fig. 1a) or sodium (Fig. 1b) abolished this pH_o change, as would be expected if it were produced by glutamate uptake⁷. Hyperpolarizing to more negative potentials, to activate more uptake, generated larger $[\text{H}^+]_o$ changes (Fig. 1c) which were proportional to the uptake current activated (Fig. 1d). D-Aspartate, a non-metabolized analogue of L-glutamate, evoked less uptake current³ and pH_o change (Fig. 1e) than glutamate, but the ratio of pH_o change to uptake current was similar for L-glutamate and D-aspartate (Fig. 1f), showing that glutamate metabolism does not cause the pH_o change.

Intracellular pH (pH_i) changes produced by uptake were monitored using the dye BCECF⁹. Glutamate evoked an intracellular acidification in voltage-clamped cells loaded with BCECF using the patch pipette (Fig. 2a), and in unclamped undialysed cells loaded⁹ with the acetoxymethyl ester of BCECF (Fig. 2b). This response had the pharmacology and Na^+ -dependence of uptake (Fig. 2b, c). D-Aspartate produced less uptake current and acidification than L-glutamate, but the ratio of pH_i change to uptake current was similar for these agents (Fig. 2d). Amiloride (1 mM), which blocks Na^+/H^+ exchange by 90% (ref. 10), and 0.5 mM DIDS, which blocks $\text{Na}^+/\text{HCO}_3^-$ co-transport by 95% (ref. 11), did not affect the pH_i change per uptake current (Fig. 2 legend), so the pH_i change was not due to uptake affecting these carriers by raising $[\text{Na}^+]_i$.

To test whether the uptake carrier transports a pH-changing anion (such as OH^- or HCO_3^-) out of the cell, rather than transporting a proton in, we replaced the normal Cl^- ions in the pipette (and thus inside the cell) with other anions. The aim was to find an anion which would replace a putative transported pH-changing anion and thus alter the size of the uptake current. Replacement of pipette Cl^- with the large anion gluconate³ or

Received 12 August; accepted 20 October 1992.

1. Schousboe, A. *Int. Rev. Neurobiol.* **22**, 1-45 (1981).
2. Kanner, B. I. & Schuldiner, S. *CRC Crit. Rev. Biochem.* **22**, 1-38 (1987).
3. Christensen, H. N. *Physiol. Rev.* **70**, 43-77 (1990).
4. Wingrove, T. G. & Kimmich, G. A. *Am. J. Physiol.* **255**, C737-744 (1988).
5. Silbermagl, S. *Klin. Wochenschr.* **57**, 1009-1019 (1979).
6. Fonnum, F. *J. Neurochem.* **42**, 1-11 (1984).
7. Nicholls, D. & Attwell, D. *Trends Pharmacol. Sci.* **11**, 462-468 (1990).
8. Rothstein, J. D., Martin, L. J. & Kuncel, R. W. *New Engl. J. Med.* **326**, 1464-1468 (1992).
9. Hediger, M. A., Coady, M. J., Ikeda, T. S. & Wright, E. M. *Nature* **330**, 379-381 (1987).
10. Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. *J. molec. Biol.* **179**, 125-142 (1984).
11. Wang, C.-D., Buck, M. A. & Fraser, C. M. *Molec. Pharmacol.* **40**, 168-179 (1991).
12. Ferkany, J. & Coyle, J. T. *J. Neurosci. Res.* **16**, 491-503 (1986).
13. Brew, H. & Attwell, D. *Nature* **327**, 707-709 (1987).
14. Cox, D. W., Headley, M. H. & Watkins, J. C. *J. Neurochem.* **29**, 579-588 (1977).
15. Robinson, M. B., Hunter-Ensor, M. & Sinor, J. *Brain Res.* **544**, 196-202 (1991).
16. Barbour, B., Brew, H. & Attwell, D. *J. Physiol., Lond.* **436**, 169-193 (1991).
17. Barbour, B., Brew, H. & Attwell, D. *Nature* **335**, 433-435 (1988).
18. Romano, P. M., Ahearn, G. A. & Storelli, C. *Am. J. Physiol.* **257**, R180-188 (1989).
19. Schwartz, E. A. & Tachibana, M. *J. Physiol., Lond.* **426**, 43-80 (1990).
20. Tolner, B., Poolman, B., Wallace, B. & Konings, W. N. *J. Bact.* **174**, 2391-2393 (1992).
21. Engelke, T., Jording, D., Kapp, D. & Puhler, A. *J. Bact.* **171**, 5551-5560 (1989).
22. Deguchi, Y., Yamato, I. & Anraku, Y. *J. Biol. Chem.* **265**, 21704-21708 (1990).
23. Wells, R. G. *et al.* *Am. J. Physiol.* **263**, F459-465 (1992).
24. Guastella, J. *et al.* *Science* **249**, 1303-1306 (1990).
25. Pacholczyk, T., Blakely, R. D. & Amara, S. G. *Nature* **350**, 350-354 (1991).
26. Kilty, J., Lorang, D. & Amara, S. G. *Science* **254**, 78-79 (1991).
27. Shimada, S. *et al.* *Science* **254**, 576-578 (1991).
28. Blakely, R. D. *et al.* *Nature* **354**, 66-70 (1991).
29. Hoffman, B., Mezey, E. & Brownstein, M. J. *Science* **254**, 579-580 (1991).
30. Smith, K. E., Borden, L. A., Hartig, P. R., Branchek, T. & Weinshank, R. L. *Neuron* **8**, 927-935 (1992).
31. Fremeau, R. T. Jr, Caron, M. G. & Blakely, R. D. *Neuron* **8**, 915-926 (1992).
32. Cotman, C. W., Monaghan, D. T., Ottersen, O. P. & Storm-Mathisen, J. *Trends Neurosci.* **10**, 273-283 (1987).
33. Eisen, A. & Calne, D. *Can. J. Neurol. Sci.* **19**, 117-123 (1992).
34. Manev, H., Costa, E., Wroblewski, J. T. & Guidotti, A. *FASEB J.* **4**, 2789-2797 (1990).
35. Siesjö, B. K. *News Physiol. Sci.* **5**, 120-125 (1990).
36. Hagberg, H. *et al.* *J. cereb. Blood Flow Metab.* **5**, 413-419 (1985).
37. Szatkowski, M., Barbour, B. & Attwell, D. *Nature* **348**, 443-446 (1990).
38. Kozak, M. *J. Cell Biol.* **115**, 887-903 (1991).
39. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. *Rev. Biochem.* **56**, 567-613 (1987).
40. Hediger, M. A., Ikeda, T., Coady, M., Gunderson, C. B. & Wright, E. M. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2634-2637 (1987).
41. Hediger, M. A. *Analyt. Biochem.* **159**, 280-286 (1986).
42. Wells, R. G. & Hediger, M. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 5596-5600 (1992).
43. Kanai, Y. *et al.* *Am. J. Physiol.* (in press).

ACKNOWLEDGEMENTS. We thank M. Stelzner for sharing the results of early uptake studies, W.-S. Lee for help with *in situ* hybridization, A. Yu for help with PCR amplification, C. Smith and S. Hebert for discussion, and P. Boutin for technical assistance. This work was supported by a grant from the National Institutes of Health to M.A.H., and by a long-term research fellowship from the International Human Frontier Science Program Organization to Y.K.

with Br^- or I^- (Fig. 3a) did not affect the uptake current. But replacing Cl^- by NO_3^- , SCN^- or ClO_4^- increased the uptake current 2- to 4-fold (Fig. 3 legend).

To test whether NO_3^- , SCN^- or ClO_4^- are indeed transported out of the cell on the uptake carrier, or merely speed uptake (for example, allosterically), we measured the uptake-evoked pH_o change with NO_3^- or ClO_4^- in the cell. If these anions merely speed uptake (and the pH change is due to H^+ transport into the cell), they should increase the uptake current and $[\text{H}^+]_o$ change proportionally. But if they are transported in place of a pH-changing anion, then, as the salts of strong acids, they should

generate no pH change and the pH_o change per uptake current should be less than with Cl^- in the cell. Experimentally (Fig. 3b), this latter prediction was found to hold for NO_3^- (5-fold less) and ClO_4^- (3-fold less; SCN^- was not tested).

Partial replacement of Cl^- with HCO_3^- also increased the uptake current (Fig. 3c), suggesting that HCO_3^- can be transpor-

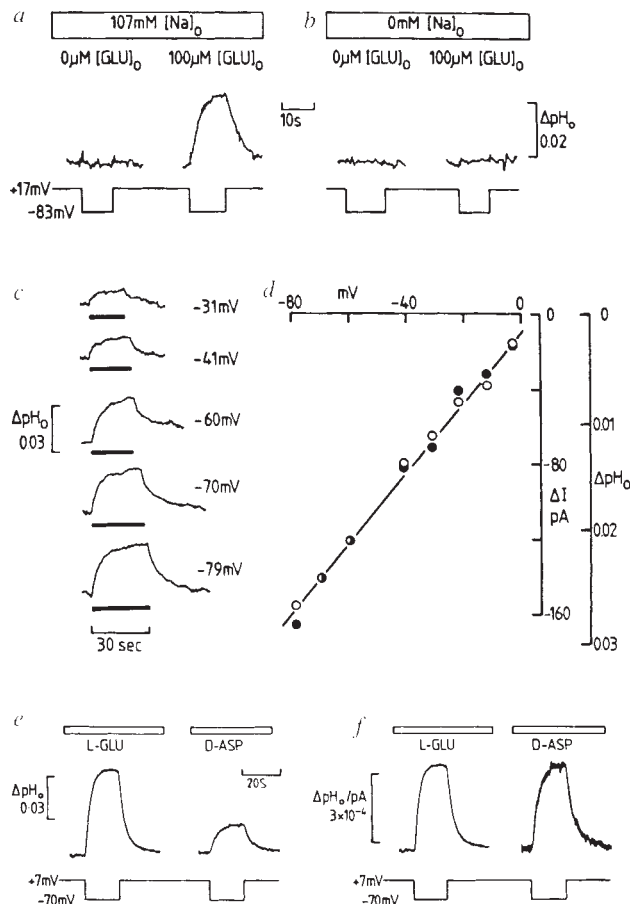


FIG. 1 Glutamate uptake generates an extracellular alkalization. *a* and *b*, Changes in pH recorded with a pH-sensitive microelectrode just outside a Müller cell (ΔpH_o , alkaline upwards) during membrane polarization from +17 mV (where uptake is small⁷) to -83 mV (where uptake is large⁷) in solutions containing 107 mM (*a*) or 0 mM (*b*) sodium and 0 μM or 100 μM L-glutamate. *c*, ΔpH_o (as in *a*) with 100 μM glutamate during activation of different amounts of uptake by polarization from +7 mV to various test potentials (bars). *d*, ΔpH_o (●), as in *c* (12 s after starting the voltage step), and the increase in uptake current (○) from its value at +7 mV in the same cell, as a function of test potential. For small changes ΔpH_o is roughly proportional to $\Delta[\text{H}^+]_o$. *e*, ΔpH_o , as in *a*, but polarizing from +7 to -70 mV, in the presence of 100 μM L-glutamate or D-aspartate. *f*, Data in *e* normalized by the extra uptake currents produced by the +7 to -70 mV voltage steps (162 and 54 pA for L-Glu and D-Asp). In 16 cells, the ratio of the $[\text{H}^+]$ changes (normalized by uptake current) evoked by D-Asp and L-Glu was 0.91 ± 0.08 (s.e.).

METHODS. Glial (Müller) cells isolated from the tiger salamander retina⁷ were whole-cell clamped using pipettes containing (mM): KCl (95); HEPES (5); NaCl (5); Na_2ATP (5); MgCl_2 (7); CaCl_2 (1); K_2EGTA (5); pH set to 7.0 with KOH. External solution contained (mM): NaCl or choline-Cl (105); KCl (2.5); CaCl_2 (3); MgCl_2 (0.5); glucose (15); BaCl_2 (6) , (to block the potassium conductance of the cells); HEPES (0.05); pH set to 7.3 with *N*-methyl-D-glucamine. Glass (Hinke type) or resin-filled (Fluka ionophore 95291) pH-sensitive microelectrodes²³ gave similar results.

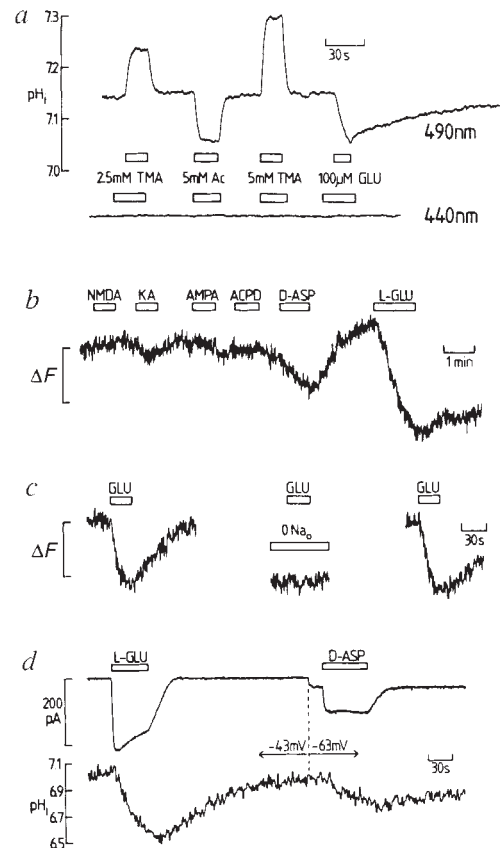


FIG. 2 Glutamate uptake generates an intracellular acidification. *a*, Changes of pH in a cell, ΔpH_i , at -43 mV, monitored with BCECF (96 μM , in the internal solution used for Fig. 1, but with 0.5 mM HEPES). External trimethylamine base (TMA) and acetic acid (Ac) (time of application indicated by open bars) increased or decreased BCECF fluorescence (at 530 nm wavelength) excited at 490 nm, owing to alkalization and acidification of the cell, but did not alter fluorescence excited at 440 nm (near the isosbestic wavelength: record obtained just after the 490 nm record from the same cell). Such data were used to calibrate²⁴ 490 nm signals: nigericin²⁵ calibration gave similar results. Mean ΔpH_i in 45 cells after 20 s of 100 μM glutamate at -43 mV was 0.23 ± 0.04 (s.e.), which is consistent with an efflux of about one OH^- ion per unitary charge of a 200 pA uptake current in a cell of volume 10^{-14} m^3 and typical buffering power of 20 mM per pH unit. Other data in this figure were obtained with 490 nm light. External solution as for Fig. 1, but 5 mM HEPES and pH set with NaOH. *b*, ΔpH_i evoked by glutamate analogues (30 μM) in an intact unclamped cell, measured as fluorescence (ΔF ; not calibrated for this cell) of BCECF, loaded by 30-min incubation in 10 μM of its acetoxymethyl ester and 0.2 μM pluronic acid⁹. The ΔpH_i produced by L-Glu, D-Asp, *trans*-ACPD, kainate, NMDA, quisqualate and AMPA had relative values ($\pm$ s.e.; number of cells in parentheses): 1, 0.47 ± 0.05 (11), 0.023 ± 0.023 (5), -0.01 ± 0.06 (7), 0 (10), 0(5) and 0(5). External Ba^{2+} was left out so that uptake would not depolarize the cell more than a few mV from its -90 mV resting potential²⁶. Mean ΔpH_i in 10 cells after 20 s of 100 μM Glu was 0.11 ± 0.02 (44 $\pm$ 9% less with 5% $\text{CO}_2/\text{HCO}_3^-$ outside). *c*, Replacing external NaCl by choline-Cl reversibly abolishes the pH_i change (measured as in *a*). *d*, Changes in pH_i (as in *a*) and uptake current produced by 100 μM L-Glu and D-Asp (applied at a more negative potential to increase its uptake current³). The ratio of ΔpH_i /uptake current evoked by D-Asp to that evoked by L-Glu was 0.98 ± 0.10 (20 cells). ΔpH_i /uptake current evoked by 100 μM Glu in 1 mM amiloride or 0.5 mM DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) was 0.95 ± 0.04 (5 cells) and 1.11 ± 0.10 (7 cells) of its value without blockers. DIDS reduced the uptake current by 49 $\pm$ 7%, probably by binding to the carrier's anion-binding site.

ted out of the cell by the uptake carrier. However, with no added HCO_3^- , inhibiting carbonic anhydrase did not affect the pH changes associated with uptake (Fig. 3*d, e*), implying that these pH changes do not reflect HCO_3^- transport (see later).

Transport of anions out of the cell on the uptake carrier was demonstrated directly using an anion-sensitive electrode just outside the cell. Micromolar levels of ClO_4^- or SCN^- could be detected in the presence of the normal extracellular chloride concentration (Fig. 4*a*). With ClO_4^- or SCN^- , but not Cl^- , as

the main anion in the cell, applying glutamate led to ClO_4^- or SCN^- accumulating outside the cell (Fig. 4*b, c*). This response had the pharmacology and sodium-dependence of glutamate uptake (Fig. 4*d, e*). The amount of ClO_4^- accumulating was proportional to the uptake current at different membrane potentials (Fig. 4*f*) and was similar to the rise in $[\text{ClO}_4^-]_o$ predicted from the uptake current flowing (Fig. 4*g* legend).

These data show that the glutamate uptake carrier generates an extracellular alkalinization and an intracellular acidification

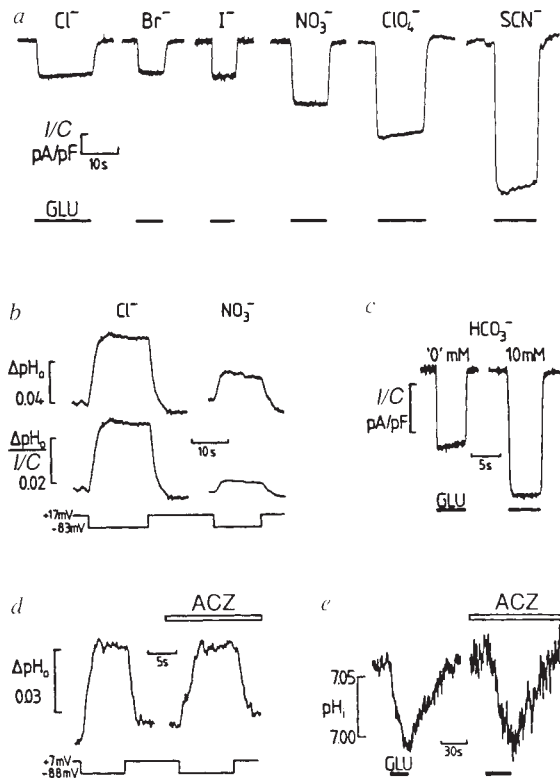


FIG. 3 The size of the glutamate uptake current depends on the intracellular anion present. Typical currents evoked at -43 mV by $30 \mu\text{M}$ Glu in cells filled with different anions. Pipettes contained (in mM): K^+ -anion (112); K_2EGTA (5); HEPES (5); pH set to 7.0 with KOH. Different cells were studied, alternating between Cl^- and a test anion in the pipette. *a*, Uptake currents (normalized by cell capacitance) with Cl^- , Br^- , I^- , NO_3^- , ClO_4^- , SCN^- had the ratio 1, 1.4 ± 0.3 (s.e.) (8 cell pairs), 0.9 ± 0.2 (3), 2.4 ± 0.3 (18), 2.8 ± 0.4 (10), 4.1 ± 0.5 (5). *b*, Top: ΔpH_o (as in Fig. 1*a*, with $30 \mu\text{M}$ Glu) outside 2 cells containing Cl^- or NO_3^- . Bottom: the same ΔpH_o normalized by uptake current/capacitance (pA/pF). In 5 cell pairs, the normalized ΔpH_o was 0.035 ± 0.005 with Cl^- , and 0.0067 ± 0.0020 with NO_3^- . For 5 cell pairs with Cl^- or ClO_4^- , values were 0.032 ± 0.006 and 0.011 ± 0.001 . *c*, Currents/capacitance evoked by $100 \mu\text{M}$ Glu at -43 mV in 2 cells, using a Cl^- internal solution as for Fig. 1 but with 1 mM acetazolamide (ACZ) and (in mM) MgCl_2 (2), Mg-ATP (5), NaCl (10) instead of MgCl_2 (7), Na_2ATP (5), NaCl (5), or the same with 10 mM NaHCO_3 /5% CO_2 replacing 10 mM NaCl . In 10 cell pairs 10 mM HCO_3^- increased the current by $39 \pm 18\%$. *d*, ΔpH_o (as in Fig. 1*a*) outside a cell (Cl^- inside) with and without 1 μM external ACZ. In 4 cells, ΔpH_o in ACZ was 1.05 ± 0.15 of its control value. *e*, ΔpH_i (as for Fig. 2*a*; Cl^- inside) with and without 100 μM external ACZ. In 6 cells, ΔpH_i in ACZ was 0.88 ± 0.07 of its control value (or 0.92 ± 0.07 in 5 unclamped cells, as for Fig. 2*b*). Normal size uptake currents were seen (as in *c*) with 1 mM internal ACZ, which blocks carbonic anhydrase ($\text{IC}_{50} \approx 10^{-8}$ M). We measured the $[\text{HCO}_3^-]$ and $[\text{CO}_2]$ in our ' HCO_3^- -free' pipette solutions as 400 and $50 \mu\text{M}$. If 1 HCO_3^- were transported per unitary charge of a 200 pA uptake current, an efflux of $2 \times 10^{-15} \text{ mol s}^{-1}$, then the $4 \times 10^{-15} \text{ mol HCO}_3^-$ in a 10^{-14} m^3 cell would be lost in 2 s. With carbonic anhydrase blocked, generation of HCO_3^- from $50 \mu\text{M CO}_2$ (rate constant²⁷ 0.037 s^{-1}) would be only $1.9 \times 10^{-17} \text{ mol s}^{-1}$, and diffusion (with coefficient $2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$) from a $3 \text{ M}\Omega$ series resistance pipette (filled with solution of resistivity $0.8 \Omega \text{ m}$) could replenish only $2.1 \times 10^{-16} \text{ mol s}^{-1} \text{ HCO}_3^-$, so HCO_3^- transport cannot be obligatory. By contrast, dissociation of water (56 M, rate constant²⁸ $2.5 \times 10^{-9} \text{ s}^{-1}$) can generate $1.4 \times 10^{-14} \text{ mol s}^{-1}$ of OH^- .

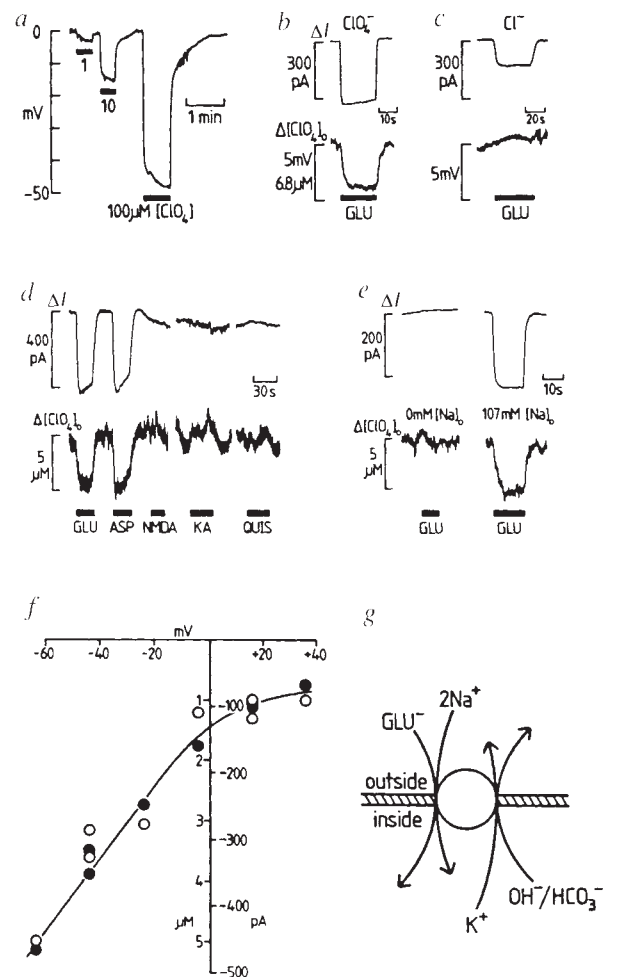


FIG. 4 The glutamate uptake carrier transports anions out of the cell. *a*, Response of an anion-sensitive microelectrode (filled with Fluka ionophore 24902) to Ringer's (as for Fig. 2) containing different $[\text{ClO}_4^-]$ (bars) to calibrate the electrode. *b* and *c*, Uptake currents at -43 mV (top) and response of an extracellular anion-sensitive electrode (bottom) evoked by $30 \mu\text{M}$ Glu in 2 cells clamped with electrodes (as for Fig. 3) containing ClO_4^- (*b*) and Cl^- (*c*). A 5 mV change of anion-sensitive electrode voltage implies a $6.8 \mu\text{M}$ rise of $[\text{ClO}_4^-]$ when ClO_4^- is present. The $[\text{ClO}_4^-]$ change per uptake current was not affected by raising $[\text{Na}^+]_{\text{pipette}}$ from 0 to 30 mM to reduce the fractional change of $[\text{Na}^+]_{\text{pipette}}$, produced by uptake, suggesting that the ClO_4^- efflux was not produced secondarily by a Na^+ -dependent anion carrier like the $\text{Na}^+/\text{HCO}_3^-$ co-transporter. *d-f*, Data from cells filled with ClO_4^- . *d*, Uptake currents at -43 mV (top) and extracellular ClO_4^- accumulation (bottom) evoked by $30 \mu\text{M}$ L-Glu, D-Asp, NMDA, kainate (KA) and quisqualate (QUIS) (data typical of 5 cells). Note that internal ClO_4^- increases the maximum velocity (V_{max}) of uptake for Asp relative to that for Glu (compare with Fig. 2*d*). *e*, Uptake current at -43 mV (top) and $[\text{ClO}_4^-]_o$ rise (bottom) evoked by $30 \mu\text{M}$ L-Glu in Ringer's with no sodium (replaced by choline), or with normal sodium. *f*, Voltage dependence of the uptake current (●) and $[\text{ClO}_4^-]_o$ rise (○) evoked by $30 \mu\text{M}$ Glu (curve drawn by eye). *g*, Proposed stoichiometry of the glutamate uptake carrier. If, with ClO_4^- inside, 64% ($1.8/2.8$ from Fig. 3*a*) of carrier cycles transport one ClO_4^- out of the cell, and the diffusion coefficient of ClO_4^- is $2 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$, then the $[\text{ClO}_4^-]$ expected at the surface of a $20\text{-}\mu\text{m}$ diameter cell for a 300 pA uptake current is $8 \mu\text{M}$, similar to a typical rise of $6 \mu\text{M}$ seen experimentally.

by transporting a pH-changing anion, presumably OH^- or HCO_3^- , out of the cell. *In vivo* some HCO_3^- transport may occur (Fig. 3c), but HCO_3^- cannot be the main anion transported in our experiments because in other cells inhibiting carbonic anhydrase greatly reduces¹² pH changes generated by HCO_3^- movement (compare Fig. 3d, e). Furthermore, with carbonic anhydrase blocked there is not enough HCO_3^- production in the cell to provide one HCO_3^- per unitary charge of the observed uptake current, whereas dissociation of water could provide enough OH^- to account for the current (Fig. 3 legend). We therefore propose the stoichiometry shown in Fig. 4g, in which uptake of one glutamate is accompanied by two Na^+ and by the counter-transport of a K^+ and an OH^- (or occasionally an HCO_3^- *in vivo*). This is consistent with the first-order dependence of uptake on external glutamate and internal potassium concentrations^{2,7}, and with the thermodynamic determination⁴ that two sodium ions are transported.

Uptake-induced changes of pH constitute a novel second messenger action of glutamate. Prolonged activation of uptake, as may occur during epileptic seizures or in the retina where glutamate is released tonically, can lower the intracellular pH significantly, and a larger alkalization is expected in the extracellular space, which is of smaller volume. Thus, the glutamate-evoked alkali flux may contribute to the changes of extra- and intracellular pH induced by neuronal activity¹³. It may also provide a signal to alter glial cell metabolism when neurons release glutamate at a high rate.

The extracellular glutamate concentration, $[\text{Glu}]_o$, is determined by the balance between the release of glutamate and its removal by uptake. However, even with no release, a lower limit to $[\text{Glu}]_o$ is set by the stoichiometry of the uptake carrier. For Fig. 4g, the minimum $[\text{Glu}]_o$ is given by $V_{\text{Glu}} = 2V_{\text{Na}} + V_{\text{OH}} - V_{\text{K}} - V_{\text{m}}$, where V_{m} is membrane potential and V_{X} is the Nernst potential of X. (This holds even if some HCO_3^- is transported, because $V_{\text{HCO}_3^-} = V_{\text{OH}}^-$.) Normally at 37 °C, with $V_{\text{Na}} = 50$ mV, $V_{\text{OH}} = -20$ mV, $V_{\text{K}} = V_{\text{m}} = -90$ mV and $[\text{Glu}]_i = 10$ mM (refs 14–16), this predicts a minimum $[\text{Glu}]_o$ of 0.6 μM . This will activate presynaptic L-AP4 receptors¹⁷ and reduce glutamate release. It will also activate some postsynaptic NMDA receptors^{18–20}, allowing some postsynaptic Ca^{2+} influx at synapses that are not releasing glutamate (when other active synapses depolarize the cell and remove Mg^{2+} -block of the NMDA channels). In anoxia, when rundown of ion gradients leads to $V_{\text{Na}} = 24$ mV, $V_{\text{OH}} = 0$ mV and $V_{\text{K}} = V_{\text{m}} = -20$ mV, $[\text{Glu}]_o$ is predicted to rise to 370 μM , which can cause neuronal death^{21,22}. □

HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides

Janice M. Riberdy*, John R. Newcomb*,
Michael J. Surman*, James A. Barbosa†
& Peter Cresswell*‡

* Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut 06510, USA

† Department of Pre-Clinical Technology, Institute of Inflammation and Autoimmunity, Miles Research Center, 400 Morgan Lane, West Haven, Connecticut 06516, USA

THE invariant chain, which associates with the major histocompatibility complex (MHC) class II molecules in the endoplasmic reticulum, serves two functions important in antigen processing. First, it prevents class II molecules from binding peptides in the early stages of intracellular transport^{1–3}. Second, it contains a cytoplasmic signal that targets the class II-invariant chain complex to an acidic endosomal compartment^{4–6}. Proteolytic cleavage and subsequent dissociation of the invariant chain then occurs^{7,8}, allowing peptides derived from endocytosed proteins to bind to released class II molecules before their expression at the cell surface³. Certain human cell lines that are mutant in one or more MHC-linked genes are defective in class II-restricted antigen processing^{9–11}. Here we show that in transfectants of one of these cell lines, T2, this deficiency results in the association of a large proportion of class II molecules with a nested set of invariant-chain-derived peptides (class II-associated invariant chain peptides, or CLIP). HLA-DR3 molecules isolated from T2 transfectants can be efficiently loaded with antigenic peptides by exposure to a low pH *in vitro*, perhaps reflecting the *in vivo* conditions in which peptides associate with class II molecules^{12–14}. Addition of synthetic CLIP inhibits the loading process, indicating that CLIP may define the region of the invariant chain responsible for obstructing the class II binding site.

Class II $\alpha\beta$ dimers isolated from certain antigen-processing mutant cell lines⁹ or from T2 transfectants¹¹ lack the characteristic stability of these molecules in SDS detergent solutions^{15–17}. Acquisition of this stability is associated with maturation of the dimers, loss of invariant chain, and binding of antigenic peptide^{13,14,18}, and it has therefore been suggested that class II molecules in the mutant cell lines are devoid of bound peptides. To test this hypothesis, HLA-DR3 $\alpha\beta$ dimers were affinity-purified from a T2 transfectant, peptides isolated from them and these peptides were separated by reversed-phase HPLC as described^{19,29}. Figure 1 shows the chromatography profiles of HLA-DR3-associated peptides from both mutant and wild-type cells. The profile from HLA-DR3 molecules purified from T2.DR3 contained a characteristic set of late-eluting peaks undetectable in an isolate of the same molecules purified from HLA-DR3 homozygous wild-type cells. These species were present in exceptionally high amounts compared to peptides in similar profiles from wild-type cells (Fig. 1; ref. 29).

The peptides associated with HLA-DR3 from T2.DR3 were sequenced (Table 1). The peaks contained peptides derived solely from a small region of the invariant chain. We determined the molecular mass of three of the peptides by mass spectrometry, which allowed a precise determination of their carboxy termini. The longest peptide based on this analysis consisted of residues 81 to 104 of the invariant chain (numbering from the

Received 14 August; accepted 13 November 1992.

- Kanner, B. I. & Schuldiner, S. *CRC Crit. Rev. Biochem.* **22**, 1–38 (1987).
- Barbour, B., Brew, H. & Attwell, D. *Nature* **335**, 433–435 (1988).
- Barbour, B., Brew, H. & Attwell, D. *J. Physiol.* **436**, 169–193 (1991).
- Erecinska, M., Wantorski, D. & Wilson, D. F. *J. Biol. Chem.* **258**, 9069–9077 (1983).
- Schwartz, E. A. & Tachibana, M. *J. Physiol.* **426**, 43–80 (1990).
- Nicholls, D. & Attwell, D. *Trends Pharmac. Sci.* **11**, 462–468 (1990).
- Brew, H. & Attwell, D. *Nature* **327**, 707–709 (1987).
- Sarantis, M. & Attwell, D. *Brain Res.* **516**, 322–325 (1990).
- Rink, T. J., Tsien, R. Y. & Pozzan, T. *J. Cell Biol.* **95**, 189–196 (1982).
- Putnam, R. W., Roos, A. & Wilding, T. J. *J. Physiol.* **381**, 205–219 (1986).
- Newman, E. A. & Aston, M. L. *Glia* **4**, 424–428 (1991).
- Kaila, K., Saarikoski, J. & Voipio, J. *J. Physiol.* **427**, 241–260 (1990).
- Chesler, M. *Prog. in Neurobiol.* **34**, 401–427 (1990).
- Berger, S. J., Carter, J. G. & Lowry, O. H. *J. Neurochem.* **28**, 149–158 (1977).
- Schousboe, A., Fosmark, H. & Hertz, L. *J. Neurochem.* **25**, 909–911 (1975).
- Kvamme, E., Schousboe, A., Hertz, L., Torgner, I. A. & Svenneby, G. *Neurochem. Res.* **10**, 993–1008 (1985).
- Forsythe, I. D. & Clements, J. D. *J. Physiol.* **429**, 1–16 (1990).
- Patneau, D. K. & Mayer, M. L. *J. Neurosci.* **10**, 2385–2393 (1990).
- Sather, W., Dieudonné, S., MacDonald, J. F. & Ascher, P. *J. Physiol.* **450**, 643–672 (1992).
- Sah, P., Hestrin, S. & Nicoll, R. *Science* **246**, 815–818 (1989).
- Choi, D. W., Maulucci-Gedde, M. & Kriegstein, A. R. *J. Neurosci.* **7**, 357–368 (1987).
- Szatkowski, M., Barbour, B. & Attwell, D. *Nature* **348**, 443–446 (1990).
- Thomas, R. C. *Ion Sensitive Intracellular Microelectrodes* (Academic, London, 1978).
- Eisner, D. A. *et al.* *Pflügers Arch.* **413**, 553–558 (1989).
- Chaillet, J. R. & Boron, W. F. *J. gen. Physiol.* **86**, 765–794 (1985).
- Mobbs, P., Brew, H. & Attwell, D. *Brain Res.* **460**, 235–245 (1988).
- Edsall, J. T. & Wyman, J. *Biophysical Chemistry* 585 (Academic, New York, 1958).
- Eigen, M. & De Maeyer, L. *Proc. R. Soc. Lond. A* **247**, 505–533 (1958).

ACKNOWLEDGEMENTS. Supported by the Wellcome Trust, M.R.C., Wolfson Foundation and the Science Plan of the European Community.

‡ To whom correspondence should be addressed.

amino terminus of the shorter p33 form²⁰). The other peptides associated with HLA-DR3 from T2.DR3 were derived from the same region by truncation of up to three amino acids at the N terminus and perhaps, based on the limited mass spectrometry data, only a single residue at the C terminus. By summing the sequencing yields for the first residue of each of the peptides shown in Table 1, we calculated that a minimum of 10% of the HLA-DR3 molecules were associated with an invariant chain peptide. This is probably a gross underestimate because it takes no account of losses incurred during peptide isolation and purification. Analysis of purified radiolabelled class II molecules from T2.DR3 (data not shown) also indicates that the majority of T2 molecules do indeed bind CLIP.

The finding that CLIP is associated with SDS-unstable class II $\alpha\beta$ dimers argues that peptide association alone does not explain the unstable phenotype. The binding of antigenic pep-

TABLE 1 Sequences of HLA-DR3-associated invariant chain peptides

Amino-acid position	81	90	100 104
Sequences*	LPKPPKPVSKMRMATPL	LPKPPKPVSKMRMATPLLMQALP	LPKPPKPVSKMRMATPLLMQALPM
	PKPPKPVSKMRMATPL	PKPPKPVSKMRMATPLMQA	
	KPPKPVSKMRMATPLLMQ		
	KPPKPVSKMRMATPLLMQALPM		

* HLA-DR3 molecules were purified from 10^{10} T2.DR3 cells and associated peptides were purified by RP-HPLC as described for Fig. 1. Invariant chain peptide sequences were determined by N-terminal Edman sequencing except for underlined amino-acid residues, which were determined by mass spectrometry.

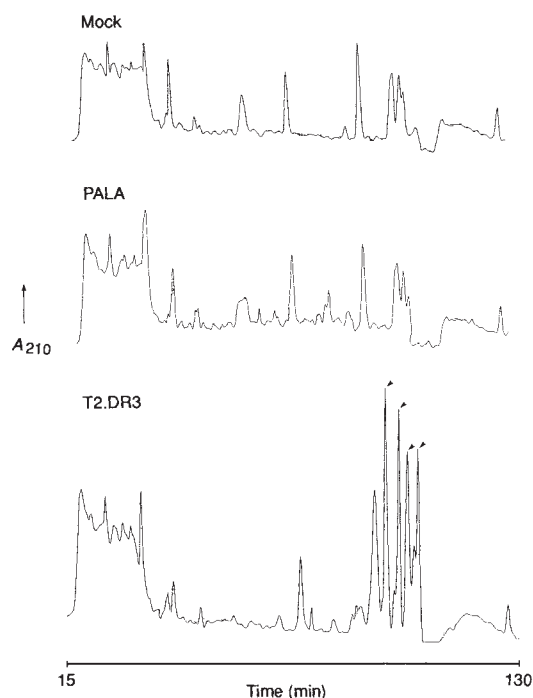


FIG. 1 Reversed phase-HPLC profiles of HLA-DR3-associated peptides from mutant and wild-type cell lines. Upper panel (Mock) shows the profile obtained from a control sample consisting of affinity column elution buffer acidified with acetic acid. Middle panel shows the HLA-DR3-associated peptides from the HLA-DR3 homozygous PALA cells. Bottom panel shows the HLA-DR3-associated peptides from T2.DR3 cells. Four peptide peaks (indicated by arrowheads) not found in the mock or wild-type PALA samples are clearly present.

METHODS. T2.DR3 transfectants (4×10^9) stably expressing HLA-DR3 and the B-LCL PALA (DR3 homozygous) (4×10^9) were lysed for 45 min at 4 °C, in 0.15 M NaCl, 0.01 M Tris, pH 7.4, containing the detergent polyoxyethylene-9-lauryl ether and proteinase inhibitors (0.1 mM *N*-tosyl-L-lysine chloromethyl ketone, 0.5 mM phenylmethylsulphonyl fluoride, and 5 mM iodoacetamide). Nuclear material was removed by centrifugation at 1,000g for 10 min and the supernatant was further clarified by ultracentrifugation at 100,000g for 1 h. HLA-DR3 molecules were purified from the cell lysates by immunoaffinity chromatography using the HLA-DR-specific monoclonal antibody L243 (ref. 28). Associated peptides were released and separated as described^{19,29}. Peptides were injected onto a μ Bondapak C18 reversed-phase HPLC column (Waters, Milford, MA) in 0.1% trifluoroacetic acid and separated using a gradient of 0% to 15% acetonitrile from 0–20 min then 15–45% acetonitrile from 20–150 min at a flow rate of 0.5 ml min⁻¹. Peptides were detected by monitoring absorbance at 210 nm (A_{210}). The four arrowheads indicate peaks present at high levels in the HLA-DR3 molecules from T2.DR3, but absent from those isolated from the wild-type PALA cells. These peaks were eluted from the reversed phase-HPLC column with retention times 96.81, 100.36, 102.65 and 105.4 min after injection.

tides to class II molecules, however, is associated with increased SDS-stability¹³, and is enhanced under acidic conditions^{12,13}. We used an *in vitro* approach¹³ to determine whether HLA-DR3 molecules isolated from T2.DR3 could be loaded with defined antigenic peptides at acid pH and whether such complexes would be stable in SDS (Fig. 2). Two peptides presented by HLA-DR3 to restricted T cells, one from the mycobacterial heat-shock protein Hsp 65 (residues 3–13)²¹ and another from the major outer member protein (MOMP) of *Chlamydia trachomatis* (residues 241–265; J.A.B., manuscript submitted), were able to generate SDS-stable complexes with HLA-DR3 from T2.DR3. The MOMP epitope was also investigated as three overlapping 15-amino-acid peptides, corresponding to residues 241–255, 246–260 and 251–265. Only the 246–260 peptide could bind and generate stable HLA-DR3 $\alpha\beta$ dimers (Fig. 2). Incubation at 37 °C with peptide for similar times without acidification did not result in the formation of stable dimers (Fig. 3, lane 3), but prolonged incubation (24 h) of T2-derived DR3 molecules at 37 °C without acidification resulted in a limited amount of stable dimer formation (data not shown).

Class II transfectants of T2 are defective in antigen processing, although they can present exogenously added antigenic peptides to class II-restricted T cells¹¹. One explanation for the defect is that these cells cannot generate stable complexes of class II molecules with peptides derived from exogenous proteins. The invariant chain-derived peptides must therefore associate by a mechanism not affected by the defect. In the mutant cells the delivery of endocytosed proteins, or the peptides derived from them²², into a post-Golgi compartment containing class II-invariant chain complexes may be impaired. A major source of peptides might then be the invariant chain, degraded by proteinases endogenous to the compartment. The invariant chain peptides would then associate with the peptide-binding groove in the same way as conventionally generated antigenic peptides, but without conferring the SDS-stable phenotype. In wild-type cells, normally processed peptides would compete with invariant chain-derived peptides for the binding site. Peptides from the same region of the invariant chain have been found in association with I-A^b (ref. 23), I-A^d (ref. 24) and HLA-DR1 (ref. 25) from wild-type cells, so the competition may not be complete. To test the ability of CLIP to bind to HLA-DR3 molecules and compete with antigenic peptides, we again used exposure to low pH to induce the formation of SDS-stable dimers. Figure 3 shows that high concentrations of CLIP (a synthetic 24-amino-acid peptide corresponding to residues 81–104 of the invariant chain) do not confer SDS stability on class II molecules purified from T2.DR3 and exposed to low pH. But at concentrations as low as 24 μ M, CLIP effectively blocked the formation of SDS-stable dimers by DR3 isolated from T2.DR3 in the presence of the antigenic peptide MOMP (246–260), whereas a control peptide at 220 μ M did not. Cycling of wild-type DR3 molecules through acidic pH in the presence of CLIP

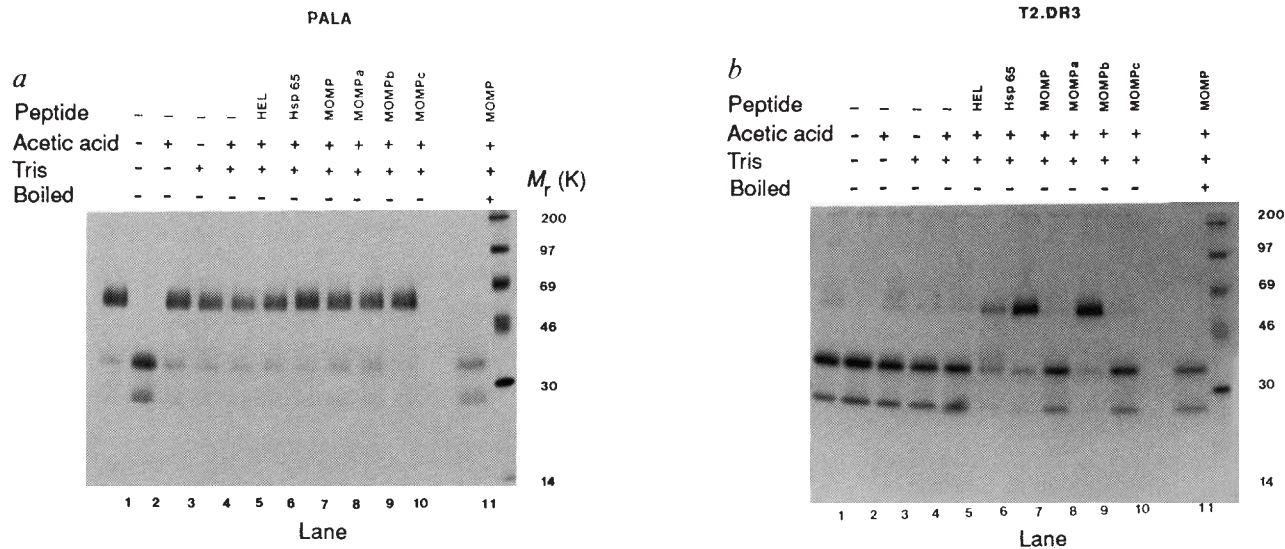


FIG. 2 Stabilization of HLA-DR3 dimers in the presence of SDS. *a*, $\alpha\beta$ -Dimers of wild-type HLA-DR3 from PALA cells remain associated in the presence of SDS under a variety of conditions. *b*, SDS-stable heterodimers of HLA-DR3 from T2.DR3 form only if the pH is lowered to 4.5 in the presence of specific antigenic peptides. Lanes: 1, mock-treated control; 2 samples reduced to pH 4.5 and not neutralized by addition of 1 M Tris; 3, samples treated with 1 M Tris only; 4–7, samples reduced to pH 4.5 in the presence of a non-specific peptide (hen egg lysozyme (HEL), residues 46–61) or specific DR3-binding peptides (Hsp65 3–13 or MOMP241–265) and neutralized by addition of 1 M Tris; 8–10, samples treated as for lanes 4–7 except that the peptides were overlapping 15-amino-acid sequences derived from MOMP241–265 (MOMP^a 241–255; MOMP^b 246–260; MOMP^c 251–265); 11, samples were

reduced to pH 4.5 in the presence of MOMP 241–265, neutralized with 1 M Tris, and heated to 100 °C before SDS-PAGE. The migration of molecular weight standards is shown on the right. METHODS. Each cell line (3×10^7 cells) was labelled for 6 h with 1.0 mCi [35 S]methionine and chased for 18 h. Class II molecules were affinity-purified as described^{1,3}. About 15,000 c.p.m. of purified $\alpha\beta$ dimers were used for each sample. Aliquots (30 μ l) were acidified from pH 7.4 to 4.5 with 3 μ l 1 M acetic acid, incubated for 60 min at 37 °C with or without peptides, and neutralized with 3 μ l 1 M Tris. All peptides were present at a final concentration of 100 μ M. An equal volume of 2 $\times$ non-reducing Laemmli sample buffer was added and samples were run on 10.5% SDS-PAGE. The samples shown in lanes 11 were heated for 5 min at 100 °C before SDS-PAGE.

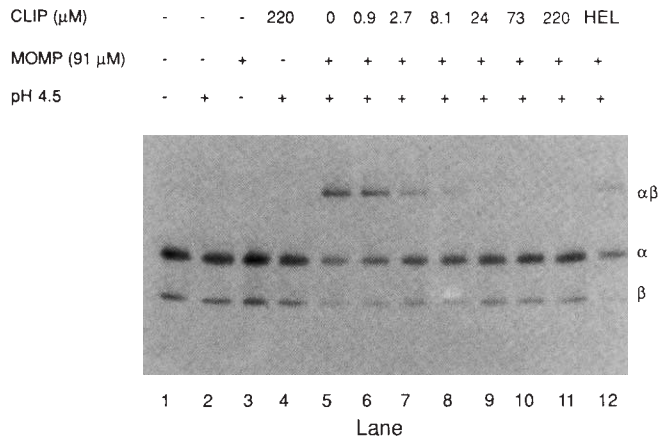


FIG. 3 CLIP blocks formation of SDS-stable dimers. Exposure to pH 4.5 and CLIP (81–104) alone does not induce SDS-stable dimers, but CLIP competes with MOMP(246–260) to block SDS-stable dimer formation. Lanes: 1, mock-treated control; 2, sample acidified to pH 4.5 and neutralized by addition of Tris; 3, sample exposed to 91 μ M MOMP(246–260) without acidification and neutralization; 4, sample acidified to pH 4.5 in the presence of 220 μ M CLIP (81–104) and neutralized with Tris; 5, sample acidified to pH 4.5 in the presence of 91 μ M MOMP(246–260) and neutralized by addition of Tris; 6–12, samples acidified to pH 4.5 in the presence of 91 μ M MOMP(246–260) and either CLIP (81–104) at the indicated concentrations, or 220 μ M HEL (46–61) and neutralized by addition of Tris. METHODS. T2.DR3 cells (1.2×10^8) were labelled for 6 h with 3.0 mCi [35 S]methionine and chased for 20 h. Class II molecules were affinity-purified as described^{1,3}. About 30,000 c.p.m. (25 μ l) of purified $\alpha\beta$ dimers were used for each sample. Samples were acidified from pH 7.4 to 4.5 with 2.5 μ l 1 M acetic acid, incubated for 60 min at 37 °C with or without peptides, and neutralized with 2.5 μ l 1 M Tris. Peptide samples were in a final volume of 41 μ l. An equal volume of 2 $\times$ non-reducing Laemmli sample buffer was added and samples were run on 10.5% SDS-PAGE.

failed to reverse their stability in SDS (data not shown). These experiments argue that CLIP binds to the HLA-DR3 molecules but fails to induce stability in SDS. This does not agree with results using truncated class II molecules²⁵ and may be due to the use of a different allele (DR1) or to alterations in structure arising from the lack of a transmembrane region or synthesis of the DR1 molecules in insect cells. A fraction of class II molecules in wild-type cells is unstable in SDS^{11,26}; these unstable dimers may contain CLIP peptides, although other peptides could be present. An alternative explanation for the origin of CLIP is that it represents a residual fragment of invariant chain that remains associated with class II molecules after proteolytic degradation. Intact human invariant chain associates with I-A^k (refs. 11, 27) as well as with HLA-DR, DQ and DP molecules, arguing that the interaction must involve highly conserved residues in the class II structure. These residues could be involved in, or sterically alter, the peptide-binding site, explaining why the invariant chain prevents association of peptides with class II molecules. Dissociation of CLIP under acidic conditions and perhaps the simultaneous association of peptides from endocytosed proteins may be the normal mechanism by which peptides associate with MHC class II molecules *in vivo*. □

Received 6 July; accepted 23 October 1992.

1. Roche, P. A. & Cresswell, P. *Nature* **345**, 615–618 (1990).
2. Teyton, L. *et al.* *Nature* **348**, 39–44 (1990).
3. Roche, P. A. & Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3150–3154 (1991).
4. Bakke, O. & Dobberstein, B. *Cell* **63**, 707–716 (1990).
5. Lotteau, V. *et al.* *Nature* **348**, 600–605 (1990).
6. Lamb, C. A., Yewdell, J. W., Bennink, J. R. & Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5998–6002 (1991).
7. Blum, J. S. & Cresswell, P. *Proc. natn. Acad. Sci. U.S.A.* **85**, 3975–3979 (1988).
8. Nguyen, Q. V., Knapp, W. & Humphreys, R. E. *Hum. Immun.* **24**, 153–163 (1989).
9. Mellins, E. *et al.* *Nature* **343**, 71–74 (1990).
10. Mellins, E., Kempin, S., Smith, L., Monji, T. & Pious, D. *J. exp. Med.* **174**, 1607–1615 (1991).
11. Riberdy, J. & Cresswell, P. *J. Immun.* **148**, 2586–2590 (1992).

12. Jensen, P. E. *J. exp. Med.* **171**, 1779–1784 (1990).
13. Sadegh-Nasseri, S. & Germain, R. N. *Nature* **353**, 167–170 (1991).
14. Germain, R. N. & Hendrix, L. R. *Nature* **353**, 134–139 (1991).
15. Billing, R. J., Safani, M. & Peterson, P. A. *J. Immunol.* **117**, 1589–1593 (1976).
16. Cresswell, P. *Eur. J. Immunol.* **7**, 636–639 (1977).
17. Springer, T. A., Kaufman, J. F., Sidioway, L. A., Mann, D. L. & Strominger, J. L. *J. biol. Chem.* **252**, 6201–6207 (1977).
18. Neefjes, J. J. & Ploegh, H. L. *EMBO J.* **11**, 411–416 (1992).
19. Wei, M. L. & Cresswell, P. *Nature* **356**, 443–446 (1992).
20. Strubin, M., Berte, C. & Mach, B. *EMBO J.* **5**, 3483–3488 (1986).
21. Geluk, A., Bloemhoff, W., DeVries, R. R. P. & Ottenhoff, T. H. M. *Eur. J. Immunol.* **22**, 107–113 (1992).
22. Harding, C. V., Collins, D. S., Slot, J. W., Geuze, H. J. & Unanue, E. R. *Cell* **64**, 393–401 (1991).
23. Rudensky, A. Y., Preston-Hurlburt, P., Hong, S. C., Barlow, A. & Janeway, C. *Nature* **353**, 622–627 (1991).
24. Hunt, D. F. *et al. Science* **256**, 1817–1820 (1992).
25. Chicz, R. M. *et al. Nature* **358**, 764–768 (1992).
26. Lanzavecchia, A., Reid, P. A. & Watts, C. *Nature* **357**, 249–252 (1992).
27. Glimcher, L. H., Polla, B. S., Poljak, A., Morton, C. C. & McKean, D. J. *J. Immunol.* **138**, 1519–1523 (1987).
28. Lampson, L. A. & Levy, R. *J. Immunol.* **125**, 293–299 (1980).
29. Newcomb, J. R. & Cresswell, P. *J. Immunol.* (in the press).

ACKNOWLEDGEMENTS. We thank K. Williams and W. McMurray for help with amino-acid sequencing and mass spectrometry, T. Buckolz for synthesis of overlapping MOMP peptides, N. Dornetios for preparing the manuscript, and A. Rudensky for discussion. The work was supported by grants from the NIH and the Howard Hughes Medical Institute.

Expression of *Hox-7.1* in myoblasts inhibits terminal differentiation and induces cell transformation

Kening Song, Yaoqi Wang & David Sassoon

Department of Biochemistry, Boston University School of Medicine,
80 East Concord Street, Boston, Massachusetts 02118, USA

THE terminal differentiation of myogenic cells initiates in the proximal portion of the limb bud whereas the distal region remains undifferentiated and proliferative^{1–3}. The apical ectodermal ridge maintains the progress zone in an undifferentiated state⁴ and induces proliferation of limb mesenchymal cells⁵. *Hox-7.1*, a homeobox-containing gene, is expressed throughout the limb bud when limb outgrowth begins, whereas transcripts are later restricted to distal limb mesenchyme^{6,7} which is the proposed site of positional specification². Transplantation of proximal limb bud tissue into the distal portion of the limb results in a re-expression of *Hox-7.1* in the transplanted mesenchyme⁸. Similar grafts result in a positional reassignment to distal structures⁹ as well as de-differentiation of the grafted proximal tissue¹⁰. Because of the association of *Hox-7.1* expression with proliferative and undifferentiated cells, we tested whether *Hox-7.1* regulates differentiation by transfection of *Hox-7.1* complementary DNA into determined myogenic cells which represent one mesenchymal lineage in the limb. Here we report that forced expression of *Hox-7.1* blocks terminal differentiation and results in a corresponding decrease in steady-state levels of MyoD1. Consistent with the association of *Hox-7.1* with proliferation, *Hox-7.1*-expressing cells also acquire a transformed phenotype. Forced expression of *Hox-8.1*, a related *Hox*-gene, does not affect terminal differentiation indicating that the effects of *Hox-7.1* are specific.

To test the effects of *Hox-7.1* on cellular differentiation and proliferation, *Hox-7.1* cDNA carried by an expression vector in either sense or antisense orientation was stably transfected into F3 myoblasts (see Fig. 1 legend). The expression of *Hox-7.1* in the transfectants was analysed by ribonuclease protection assays (Fig. 1). Whereas *Hox-7.1* transcripts are not detected in F3 parental and antisense transfectants, high levels of *Hox-7.1* transcripts are detected in all sense transfectants. Clones 1-c and 1-d express the predicted-size transcript, whereas additional truncated transcripts are detected in clones 1–4 and 1–5. The precise origin of the truncation is unknown, although it could result from a recombination event, or reflect premature termination of transcription (Fig. 1).

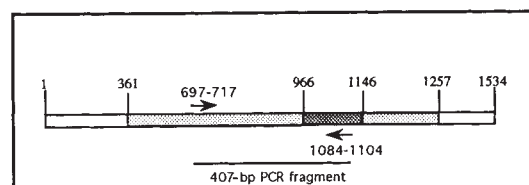
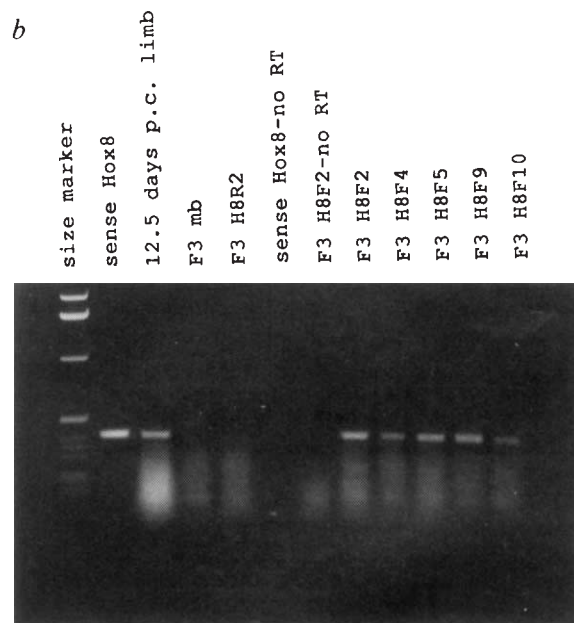
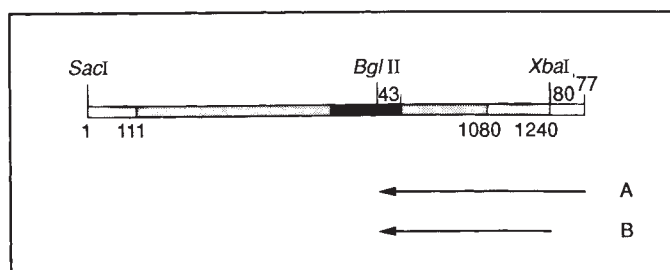
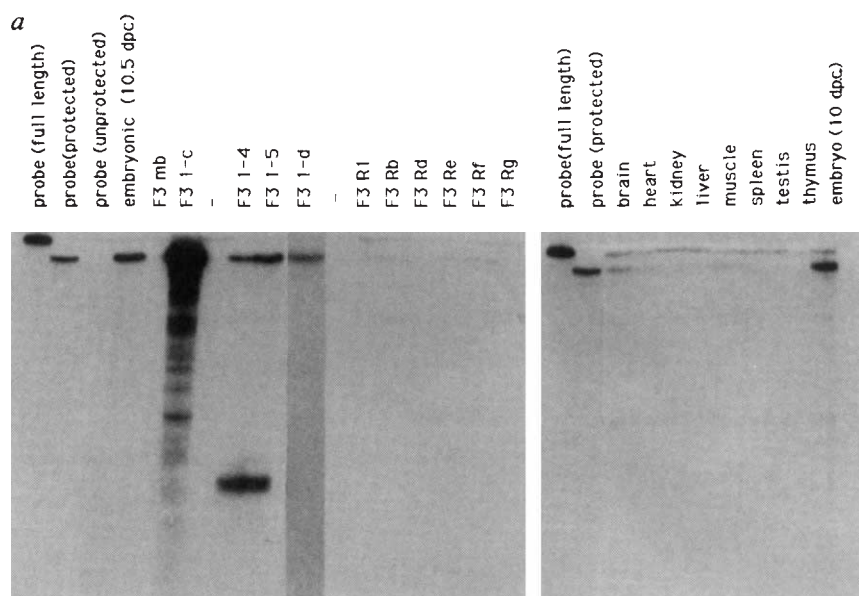
Myogenic cells will differentiate when media conditions are changed from high serum to low serum. Myoblasts typically respond by growth arrest, cellular alignment and subsequent fusion which is accompanied by an accumulation of transcripts encoding contractile proteins¹¹. When terminal differentiation is induced, F3 cells transfected by neomycin-resistant plasmid alone (F3neo), as well as antisense clones, show numerous myotubes (Fig. 2). In contrast, clone 1-c, which expresses the highest level of *Hox-7.1* (Fig. 1), fails to form myotubes (Fig. 2). Clone 1-d, which expresses lower levels of *Hox-7.1* (Fig. 1) forms myotubes at a very low frequency (Fig. 2m). The situation for clones 1–4 and 1–5 is very different. We note that these clones produce truncated transcripts in addition to the predicted size *Hox-7.1* transcript (Fig. 1). No myotube formation is observed by 24 h after differentiation in both clones, whereas small myotubes which contain one to three nuclei begin to appear by 48 h after differentiation (Fig. 2). In all cases, *Hox-7.1* expression results in blocked or delayed terminal differentiation. To test whether the effects of *Hox-7.1* were specific, we transfected cells with *Hox-8.1* which shares strong homology in the homeobox domain but is divergent in the carboxy and amino portions of the predicted protein product¹². Cells were selected using identical conditions used for the *Hox-7.1*-transfected cells, and assayed for *Hox-8.1* transcription by RNA polymerase chain reaction (RNA PCR) (Fig. 1b). In these experiments, all of the clones isolated showed normal differentiation indistinguishable from controls and the parental cell line (Fig. 2). Thus, the effects of *Hox-7.1* are specific to this member of the *msh-Hox* gene family.

We tested whether the failure to differentiate in clones 1-c and 1-d reflected transcriptional changes in one or more of the myogenic factors that mediate myogenesis. Because F3 myoblasts express high levels of MyoD1, lower levels of myogenin and barely detectable levels of myf5 and MRF4 (unpublished data), we determined whether steady state MyoD1 levels were altered in the *Hox-7.1*-expressing lines (Fig. 3). Clone 1-c, which expresses the highest levels of *Hox-7.1*, does not show detectable levels of MyoD1 messenger RNA and clone 1-d has barely detectable levels. We conclude that transcription of *Hox-7.1* can therefore block differentiation of F3 cells and may do so by a regulatory chain of events involving MyoD1. Clones 1–4 and 1–5 show normal levels of MyoD1 which is also consistent with the observation that some myotube formation occurs, although the process is delayed by about 24 hours in these clones. The presence of the truncated transcripts seems to interfere with the antagonistic effects of *Hox-7.1* on the myogenic programme. Although myotube formation is a strong indicator of terminal differentiation, we further analysed the degree of differentiation by immunostaining with an antibody to sarcomeric myosin heavy chain (MHC; Fig. 2). By 48 hours in low serum, more than 45% of cell nuclei are incorporated into MHC-positive cells in the antisense, F3neo controls, and *Hox-8.1*-transfected cells. In contrast, there is nearly a complete absence of MHC-positive cells in clone 1-c (less than 0.1%) and all the positive cells appear mononucleated. In all other *Hox-7.1*-expressing clones, less than 5% of the nuclei are incorporated into MHC-positive cells. These data confirm that *Hox-7.1* but not *Hox-8.1* expression interferes with the normal programme of differentiation in myogenic cells.

Clones 1–4 and 1–5 show a recognizable change in cell morphology. In particular, cells are more refractile and less flattened than the control F3 cells suggesting cellular transformation has occurred (see Fig. 2). The capacity of cells to grow in soft agar was tested as one measure of cell transformation¹³. Colony formation is either absent or occurs at a very low frequency in the control cells (F3neo and antisense clones) whereas all four *Hox-7.1*-transcribing clones show a much higher frequency of colony formation (Fig. 4a–d). Clones 1–4 and 1–5 show the highest frequency of colony formation which may reflect the presence of the additional truncated *Hox-7.1* transcripts

FIG. 1 a, *Hox-7.1* is expressed in sense transfectants but not in controls. *Hox-7.1* cDNA was subcloned into pEMSV scribe α 2 expression vector in both sense (SPHox-7.1) and antisense (SPHox-7R) orientations and transfected into F3 myoblasts with pSV2neo. Ribonuclease protection assays were done to detect the levels of *Hox-7.1* in the transfectants (upper panel). Sense transfectants (F31-c, 1-d, 1-4 and 1-5) show levels that are either comparable or higher than those observed in the 10 or 10.5 days post-coitum (p.c.) embryo whereas neither of the clones transfected with the antisense construct (F3R1, Rb, Rd, Re, Rf and Rg) nor the parental F3 cells show detectable levels of *Hox-7.1* transcription. Of the adult tissues examined, only brain shows low but detectable levels of *Hox-7.1* transcription. The levels of *Hox-7.1* transcripts in clone 1-c is the highest among all the transfectants. Clones 1-4 and 1-5 transcribe the full-length *Hox-7.1* mRNA and truncated transcripts. The truncation is most probably within the 3' portion of the mRNA giving rise to a sequence that is ~310 nucleotides shorter. Thus the predicted protein encoded by the truncated *Hox-7.1* mRNA will have an intact homeobox, but lack half of the cysteine-rich domain, which has been proposed to be involved in protein-protein interactions⁶. A schematic view of *Hox-7.1* cDNA and RNA probe is shown in the lower panel. Arrow A corresponds to the full-length (577 nucleotides) antisense probe, and arrow B the protected fragment with a predicted size of 497 nucleotides. **b**, *Hox-8.1* is expressed in sense transfectants but not in controls. *Hox-8.1* cDNA was subcloned into pEMSV scribe α 2 and transfected into F3 myoblasts and clones were isolated. RNA polymerase chain reaction (RNA PCR) was used to detect *Hox-8.1* transcripts in the resultant clones. RNA from 12.5 days p.c. mouse limb buds and synthetic sense *Hox-8.1* RNA (lane sense) give a PCR product of the predicted size (407 base pairs). Five sense transfectants (H8F2, H8F4, H8F5, H8F9 and H8F10) reveal the band of the predicted size whereas the *Hox-8.1* band is not seen in the F3 parental cells (F3 mb) and antisense transfectant (H8R2). The band is also not present in RNA PCR products of sense *Hox-8.1* RNA and RNA from H8F2 when reverse transcriptase is not included in the reaction (lanes sense Hox8-no RT and H8F2-no RT). The lower panel shows a schematic view of *Hox-8.1* cDNA, and the positions of the PCR primers and the 407-base pair RNA PCR product are indicated.

METHODS. **a**, Cell culture and transfection: Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (growth medium (GM)). Transfection was done as described elsewhere²¹. For each transfection, 20 μ g of the construct and 400 ng of the pSV2neo (molar ratio was 50:1) was used. Individual clones were isolated with cloning rings after G418 selection (800 μ g ml⁻¹). The F3 clones transfected by pSV2neo alone (F3neo) were also selected. All media and supplements were purchased from Gibco/BRL. Fetal bovine serum was purchased from Sigma. Ribonuclease protection assay: Total RNA was isolated using RNeasy (Qiagen/Biotech) according to the manufacturer's instructions. Antisense RNA probe of *Hox-7.1* (577 nucleotides) was synthesized in a reaction mixture containing 500 μ M of each of ATP, GTP and UTP; 2.5 μ M of CTP; 125 μ g ml⁻¹ of BSA; 50 μ Ci ³²P CTP (3,000 Ci mmol⁻¹, New England Nuclear); 1x transcription buffer, 0.5 μ g of template DNA, and 19 U of T7 polymerase in a total volume of 20 μ l at 37 °C for 30 min. The template was digested by RQ DNase at 37 °C for 15 min. For each assay, 10 μ g of total RNA was incubated with 2 $\times$ 10⁵ decays per min of probe in 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% deionized formamide at 42–45 °C for 14–18 h. Samples were then digested with 5 U ml⁻¹ RNase A and 150 U ml⁻¹ RNase T1 for 30 min at 37 °C. After phenol-chloroform extraction, samples were resolved on a 5% denaturing polyacrylamide gel. Non-radiolabelled sense *Hox-7.1* RNA was synthesized using T3 RNA polymerase in a reaction mixture containing 500 μ M of each ribonucleotide. **b**, *Hind*III–*Eco*RI fragment of *Hox-8.1* cDNA was subcloned into pEMSV scribe α 2 vector as described in Fig. 1a legend. RNA PCR was done using a kit from Perkin Elmer Cetus according to supplier's conditions. For each reaction, 1 μ g of total RNA from different cells and tissues were used. For the sense control lane, 0.2 ng of synthetic *Hox-8.1* RNA was used. All amplifications were done for 35 cycles at an annealing temperature of 60 °C. Sense *Hox-8.1* RNA was synthesized as described for **a**.

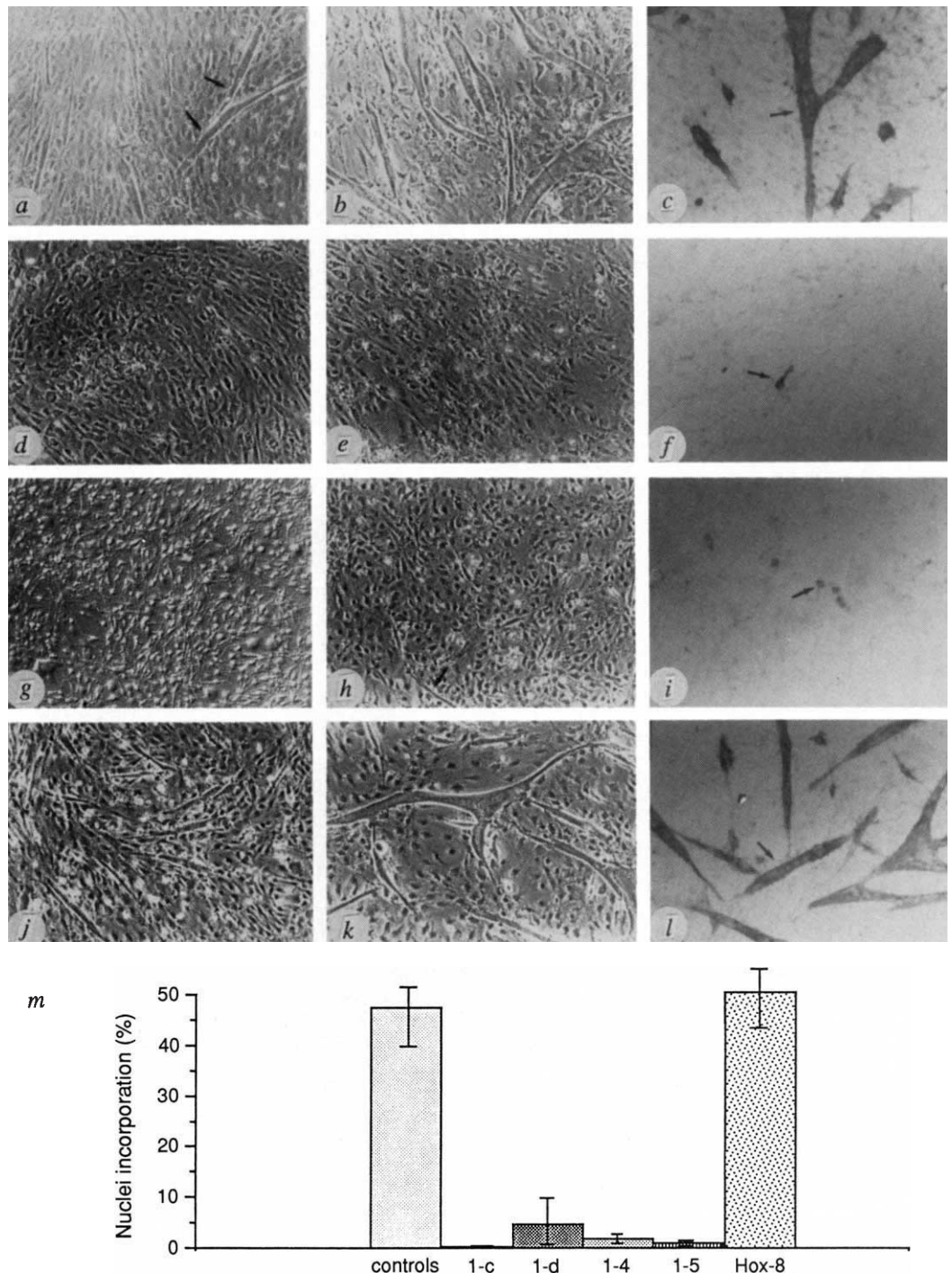


(Fig. 1). We conclude that *Hox-7.1* confers the capacity to grow in an anchorage-independent manner suggesting that *Hox-7.1* has oncogenic potential. All transfectants were tested further for tumorigenic potential by subcutaneous injection into nude mice. None of the control lines form tumours up to 5 months

whereas clone 1-5, which has the highest rate of growth in soft agar, results in tumours at all sites of injection within 2.5 months (Fig. 4e). The other *Hox-7.1*-expressing clones (1-c, 1-d, 1-4) did not produce observable tumours. The tumours from clone 1-5 appear to be fibrosarcomas and do not show overt signs of

FIG. 2 Terminal differentiation is inhibited or delayed in transfectants expressing *Hox-7.1* but not *Hox-8.1*. Cells were assayed in differentiation medium (DM) for their myogenic potentials. Myotube formation was scored at 24 h (a, d, g, j) and 48 h (b, e, h, k) by phase-contrast microscopy. Immunostaining of myosin heavy chain was done at 48 h (c, f, i, l), and the percentages of nuclei incorporated into myosin heavy chain-positive cells were determined (m). F3neo cells (a, b, c), F3 R1 (j, k, l) and all other antisense transfectants, show robust myotube formation by 24 h and 48 h (see arrow). Clone F3 1-c does not form any myotubes at the same time point viewed by phase microscopy (d, e), and immunostaining reveals very few myosin heavy chain-positive cells which are mononucleated (f arrow, m). Clone 1-d shows a very low frequency of myotube formation (m). Clone 1-5 which expresses truncated *Hox-7.1* transcripts (Fig. 1), shows no myotubes by 24 h (g), and small myotubes (see arrow) which contain one to three nuclei by 48 h (h, i see arrow). Apparent morphological changes are also observed in this clone (g) such that cells have numerous processes. Another clone (F3 1-4) which also contains truncated transcripts of *Hox-7.1* has a similar morphology and behaves similarly to F3 1-5 in response to DM (m). All *Hox-8.1*-transfected cells showed no change in either morphology nor in myotube formation and biochemical differentiation as determined by immunostaining (m, lane 'Hox-8' combined results for H8F2,4,5,9,10).

METHODS. Transfectants were seeded at the same density in 6-well plates (Fisher) in DMEM supplemented with 15% fetal bovine serum (Sigma) and $200 \mu\text{g ml}^{-1}$ of G418. To initiate differentiation, subconfluent cells were washed once with PBS and media was replaced with DMEM supplemented with 2% horse serum (DM) without G418. Immunostaining of myosin heavy chain: Cells were grown in multi-well Lab-Tek slides (Nunc) and differentiated as described above. Myosin heavy chain was assayed as described elsewhere²² with the following modifications. Cells were extracted with 0.1% Triton X-100 in PBS for 30 s, followed by fixation in 100% methanol for 1 min. After washing with 0.1% BSA in PBS, cells were reacted with the



monoclonal antibody against myosin heavy chain (MF20) at 4 °C overnight. Samples were reacted with a biotinylated goat antibody against mouse IgG followed by incubation with biotinylated horseradish peroxidase using supplier's conditions (Vectastain). Two separate assays were done for each cell line. At least 10 random fields were selected containing at least 100 nuclei. Results are presented as number of nuclei in MHC-positive cells per total number nuclei counted $\times 100$ and means and range are indicated. Results from *Hox-8.1* transfected cells have been combined as no differences were noted between clones and controls.

differentiation on histological examination (Fig. 4f). It was recently reported that *Hox-2.4* is transcriptionally activated in the mouse myeloid leukaemia WEHI-3B cell line and forced expression of this gene in NIH-3T3 cells produced fibrosarcomas in nude mice¹⁴. Another study demonstrated that *Hox-11* is up-regulated in a T-cell acute lymphoblastic leukaemia by chromosomal translocation¹⁵. Our data provides another example of Hox gene-mediated cell transformation, indicating

that Hox genes have oncogenic potential and ectopic or deregulated expression may result in tumorigenesis.

Hox-7.1 may act through a number of regulatory factors to interfere with terminal differentiation. These include growth factors, receptors¹⁶⁻¹⁸, and the myogenic factors¹⁹. The effects of forced *Hox-7.1* expression on MyoD1 in this study suggest

FIG. 3 Northern analysis of MyoD1 mRNA in transfectants expressing *Hox-7.1* transcripts. Parental (F3), F3neo and *Hox-7.1* sense (1-c, 1-d, 1-4, 1-5) and antisense (R1, Re, Rg) transfectants were grown in GM and steady-state levels of MyoD1 mRNA were analysed using northern blot analysis (upper panel). MyoD1 levels are downregulated in clones which transcribe only the predicted-size *Hox-7.1* mRNA. In clone 1-c, MyoD1 is below detectable levels and clone 1-d shows barely detected levels. Clones 1-4 and 1-5 show levels of MyoD1 mRNA similar to F3neo and antisense transfectants (R1, Re, Rg). The lower panel shows the results from the same membrane rehybridized with a β -actin probe to verify the efficiency of RNA transfer.

METHODS. Total RNA was isolated as described in legend to Fig. 1. RNA samples (10 μ g per lane) were run on a 1% agarose-3% formaldehyde gel. Transfer, hybridization and washing were done as described previously²¹. ³²P-labelled probes were synthesized by random priming (Boehringer Mannheim). The exposure time for MyoD1 was 4.5 days and for β -actin was 16 h.

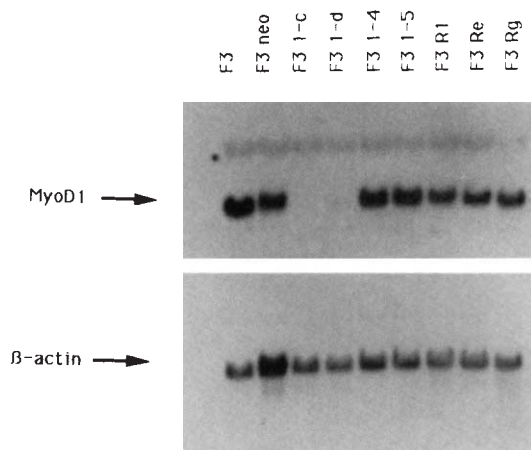
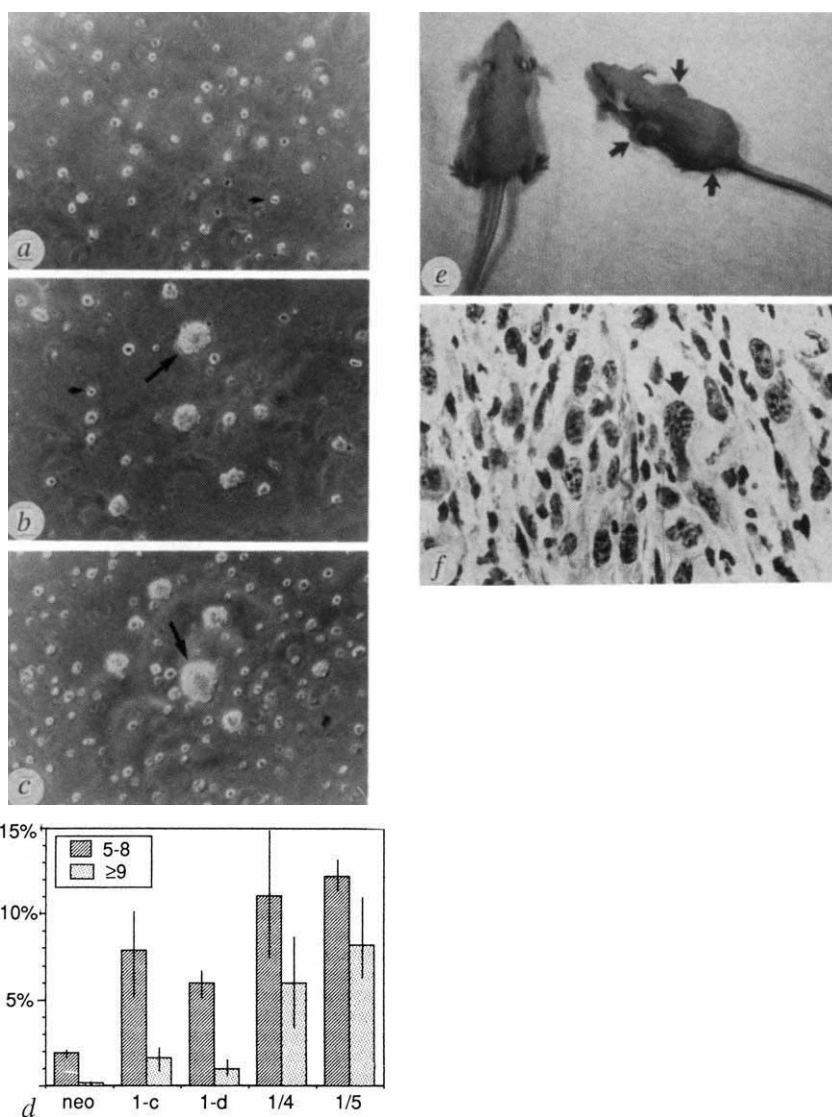


FIG. 4 *Hox-7.1* expression confers anchorage-independent cell growth and induces tumours in nude mice. A soft agar assay was used to detect the colony formation of the individual transfectants (left panel). The majority of F3neo cells (shown here in (a)) and antisense transfectants undergo growth arrest and remain as single-cell colonies (small arrow) or minute colonies containing 2 to 3 cells, whereas all sense transfectants form large colonies (large arrows) containing many tightly packed cells (shown here clones 1-c (b) and 1-5 (c)). d, Bar graph representing the percentages of cells that form colonies. Each experiment was done twice and at least 500 colonies (single or multi cell) were counted. Means $\pm$ range are indicated. Nude mice assay (e, f): F3neo and all sense and antisense transfectants were injected subcutaneously into nude mice at the three positions shown. F3 1-5 showed pronounced tumour formation. e, Arrows indicate three sites of injection at the mouse on right. Shown on left is a nude mouse injected with F3neo cells which did not result in tumour formation. f, Haematoxylin-eosin stained section of the tumour from the mouse in panel e. Many enlarged nuclei are present (see arrow); there are no signs of overt differentiation. The morphology is consistent with a fibrosarcoma. METHODS. Soft agar assays were based on the protocol described elsewhere¹³. Cells (2×10^3 – 1×10^4) were suspended in 4 ml of 0.5% low melt agarose (Boehringer Mannheim) in DMEM supplemented with 20% fetal bovine serum and were plated onto a solidified base layer of the same composition in a 60 mm dish. Plates were scored for colony formation by microscopic examination after 2 weeks. Nude mice assay: Cells were suspended in PBS. 1×10^6 , 2×10^5 , and 5×10^4 cells were injected subcutaneously into 3 positions at forequarters (left and right) and hind-quarters (left only) of nude mice, respectively. Mice were examined for tumour formation up to 5 months after injection. Mice were killed and tumours removed and fixed in 4% paraformaldehyde in PBS. Tissue was then dehydrated and embedded into JB-4 resin, sectioned on a ultramicrotome (1 μ m thickness), stained with haematoxylin-eosin and photographed.



a potential molecular explanation for the spatial regulation of differentiation during limb development *in vivo*. The accumulation of myogenin and MyoD1 does not occur in the early limb bud when outgrowth is initiated even though migratory somite-derived cells destined to form the musculature of the limb are already present in the somatopleure before the formation of the limb bud^{3,20}. Myogenic factors are first detectable in the proximal region of the limb where *Hox-7.1* transcripts are no longer present^{3,7}. The decrease in levels of MyoD1 mRNA in *Hox-7.1*-transfected cells is consistent with the observation that muscle differentiation and *Hox-7.1* expression *in vivo* are mutually exclusive. These observations lead to the hypothesis that *Hox-7.1* promotes cell proliferation and suppresses cell

differentiation *in vivo*, which is supported by the data presented in this study. Whether similar molecular mechanisms underlie the regulation of other mesenchymal cell types that display spatial gradients of differentiation (for example cartilage) during limb development awaits further investigation. *Hox-8.1*, which is expressed at high levels in the limb apical ectoderm, and at lower levels in the limb mesenchyme does not affect terminal differentiation in our assays. Thus we conclude that the molecular actions of these two highly related genes are quite different. Because the *Hox-7.1* and *-8.1* proteins share nearly perfect identity in the homeodomain, their different effects presumably arise from the divergent amino and carboxy portions. □

Received 1 September; accepted 30 September 1992.

- Kieny, M., Pautou, M. P., Chevallier, A. & Mauger, A. *Bibl. Anat.* **29**, 65–90 (1986).
- Summerbell, D. & Wolpert, L. *Nature New Biol.* **238**, 24–25 (1972).
- Sassoon, D. *et al. Nature* **341**, 303–307 (1989).
- Globus, M. & Vethary-Globus, S. *Differentiation* **6**, 91–96 (1976).
- Solursh, M., Singley, C. T. & Reiter, R. S. *Dev. Biol.* **86**, 471–482 (1981).
- Hill, R. E. *et al. Genes Dev.* **3**, 26–37 (1989).
- Robert, B., Sassoon, D., Jacq, B., Gehring, W. & Buckingham, M. *EMBO J.* **8**, 91–100 (1989).
- Davidson, D. R., Crawley, A., Hill, R. E. & Tickle, C. *Nature* **352**, 429–431 (1991).
- Saunders, J. W. Jr, Gasseling, M. T. & Cairns, J. M. *Dev. Biol.* **1**, 281–301 (1959).
- Hampé, A. *J. Embryol. exp. Morph.* **8**, 241–245 (1960).
- Buckingham, M. E. *et al. in Molecular Biology of Development* Vol. 19 (eds E. H. Davidson & R. A. Firtel) 275–292 (Liss, New York, 1984).
- Monaghan, A. P. *et al. Development* **112**, 1053–1060 (1991).
- Macpherson, L. & Montagnier, L. *Virology* **23**, 291–294 (1964).
- Aberdam, D., Negreanu, V., Sachs, L. & Blatt, C. *Molec. cell. Biol.* **11**, 554–557 (1991).
- Hatano, M., Robert, C. W. M., Minden, M., Crist, W. M. & Korsmeyer, S. J. *Science* **253**, 79–82 (1991).

- Moore, J. W., Dionne, C., Jaye, M. & Swain, J. L. *Development* **111**, 741–748 (1991).
- Brennan, T. J., Edmondson, D. G., Li, L. & Olson, E. N. *Proc. natn. Acad. Sci. U.S.A.* **88**, 3822–3826 (1991).
- Olwin, B. B. & Hauschka, S. D. *J. Cell Biol.* **107**, 761–769 (1988).
- Weintraub, H. D. *et al. Science* **251**, 761–766 (1991).
- Chevallier, A. *Wilhelm Roux Arch. Entwickl.* **184**, 57–73 (1978).
- Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning. A Laboratory manual* 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
- Bader, D., Masaki, T. & Fischman, D. A. *J. Cell Biol.* **95**, 763–770 (1982).

ACKNOWLEDGEMENTS. We thank R. Hill for many helpful conversations and for the kind gift of the p*Hox-7* and *Hox-8* plasmid. A. Lassar for encouragement and for the pEMSV scribe expression vector in addition to the F3 cell line; N. Rosenthal, S. Farmer, G. Marazzi, P. Soriano and K. Muneoka for consultation in preparing this manuscript; D. Bader for providing MF20 antibody and J. Linecum for help in immunohistochemistry. This study was supported in part by grants from The American Cancer Society and Council for Tobacco Research.

Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes

Jonathan Fawcett*, Claire L. L. Holness*,
Lindsey A. Needham†, Helen Turley*‡,
Kevin C. Gatter‡, David Y. Mason‡
& David L. Simmons*§

* ICRF Laboratories, Institute of Molecular Medicine, University of Oxford, Oxford, OX3 9DU, UK

† British Biotechnology, Cowley, Oxford OX4 5LY, UK

‡ Nuffield Department of Pathology, John Radcliffe Hospital, Oxford, OX3 9DU, UK

THE co-ordinated function of effector and accessory cells in the immune system is assisted by adhesion molecules on the cell surface that stabilize interactions between different cell types¹. Leukocyte function-associated antigen 1 (LFA-1) is expressed on the surface of all white blood cells² and is a receptor for intercellular adhesion molecules (ICAM) 1 and 2 (ref. 3) which are members of the immunoglobulin superfamily^{4–6}. The interaction of LFA-1 with ICAMs 1 and 2 provides essential accessory adhesion signals in many immune interactions, including those between T and B lymphocytes⁷ and cytotoxic T cells and their targets^{8,9}. In addition, both ICAMs are expressed at low levels on resting vascular endothelium¹⁰; ICAM-1 is strongly upregulated by cytokine stimulation and plays a key role in the arrest of leukocytes in blood vessels at sites of inflammation and injury. Recent work has indicated that resting leukocytes express a third ligand, ICAM-3, for LFA-1 (refs 11, 12). ICAM-3 is potentially the most important ligand for LFA-1 in the initiation of the immune response because the expression of ICAM-1 on resting leukocytes is low. We report the expression cloning of a complementary DNA, pICAM-3,

encoding a protein constitutively expressed on all leukocytes, which binds LFA-1. ICAM-3 is closely related to ICAM-1, consists of five immunoglobulin domains, and binds LFA-1 through its two N-terminal domains.

To identify constitutively expressed LFA-1 ligands, monoclonal antibodies raised against human leukocytes were selected on the basis of three criteria: a pan-leukocytic reactivity profile, high expression on unstimulated leukocytes, and no assignment to an existing cluster of differentiation (CD 1–75; ref. 13). One monoclonal antibody, KS128, demonstrated high expression on freshly prepared peripheral blood lymphocytes (PBL), monocytes and granulocytes (data not shown) and was selected for further study.

cDNA expression libraries constructed from U937 and Daudi cell lines were screened with KS128 antibody and a single clone with a 1.7-kilobase (kb) insert was isolated by three rounds of COS cell transfection, panning and episomal rescue¹⁴. A protein with $M_r \sim 110K$ was immunoprecipitated from COS cell transfectants and from the promonocyte cell line U937 (Fig. 1). A control anti-ICAM-1 antibody precipitated a 90K protein from ICAM-1 transfectants and from U937 cells.

RNA expression analysis (Fig. 2) reveals a single 1.7-kb transcript in most leukocytic cell lines, but most prominently in those of B-lymphocytic lineage and those with myeloid properties, such as U937 cells. Expression was low or absent in cell lines of more primitive phenotype like the promyelocytic line HL60 and those with erythroid features such as the erythro-leukaemic line K562. No endothelial cell expression was detected even after activation with interleukin IL-1 β and TNF- α .

The cDNA insert consists of 1,739 nucleotides (Fig. 3a) and has a poly(A)⁺ tail. A single 1,641-base pair (bp) open reading frame gives a predicted polypeptide sequence of 554 residues and has the typical features observed in a type I integral membrane protein. A potential initiating methionine at nucleotide position 11 (ref. 15), is followed by a putative signal peptide which may be cleaved between Gly 26 and Glu 27 (ref. 16), leaving 450 residues in the extracellular domain of the mature protein. A stretch of 25 hydrophobic residues (Phe 480 to Phe 504) is a likely transmembrane region and there are 37 residues in the cytoplasmic domain. The difference between the

§ To whom correspondence should be addressed.

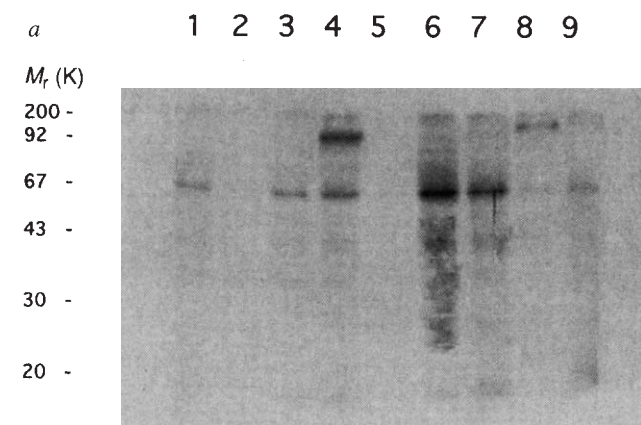


FIG. 1 Immunoprecipitation analysis of KS128 antigen. *a*, Immunoprecipitations were done with control monoclonal antibody 7B4 (lanes 1, 2, 3); anti-ICAM-1 antibody (11C8; British Biotechnology) (lanes 4, 5, 6); or antibody KS128 (lanes 7, 8, 9) from surface-iodinated COS-1 cells transfected with pICAM-1 (lanes 1, 4, 7), KS128 cDNA (lanes 2, 5, 8), or pCDM8 vector only (lanes 3, 6, 9). *b*, Phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate; TPA)-induced U937 cells precipitated with: lane 1, KS128; lane 2, 11C8 (anti-ICAM-1); lane 3, 7B4 (negative control). Cells (10^7) were surface-iodinated with iodogen (Pierce) according to the vendor's instructions, washed and lysed; lysates were pre-cleared before immunoprecipitating with 10 μ g antibody and agarose-coupled goat anti-mouse IgG (Sigma). After washing, samples were resolved on 10% SDS-PAGE and autoradiographed.

predicted core peptide M_r of 59K and that observed on immunoprecipitation (110K) indicates that many of the 15 potential *N*-linked glycosylation sites (Asn-X-Ser/Thr) are utilized.

A search of the National Biomedical Research Foundation protein database disclosed a strong homology to human ICAM-1

and -2 and the intervals between cysteine residues are conserved between the presented sequence and ICAM-1. In view of the degree of homology this cDNA has been named ICAM-3 and is predicted to encode a member of the immunoglobulin superfamily with five C2 domains. Multiple alignments of ICAM-1, ICAM-2 and ICAM-3 (Fig. 3*b*) reveal that ICAM-3 is most closely related to ICAM-1, with an overall 47% amino-acid identity rising to 77% in domain II. The least sequence conservation between the ICAMs is observed in the cytoplasmic region. Recent work delineating the LFA-1 binding sites in the first two domains of ICAM-1 (refs 17, 18) indicate the importance of the residues Asp 26, Glu 34, Gly 46 and Gln 73 in domain I. With the exception of the first, these residues are found in ICAM-3. Two (Asn 175 and Ser 177) of the four key residues in domain II are conserved in ICAM-3.

The distribution and structural data strongly suggested the possibility that ICAM-3 would be a third ligand for LFA-1 and experiments were undertaken to test this hypothesis. Specific adhesion of the promonocytic LFA-1⁺ cell line THP-1 to ICAM-1- and ICAM-3-transfected COS cells was observed (Fig. 4*a*). Binding was inhibited by an anti-LFA-1 blocking antibody specific for CD11a, monoclonal antibody 38 (ref. 19) (Fig. 4*a*). Antibody KS128 did not inhibit ICAM-3/LFA-1 adhesion (data not shown).

To assess LFA-1 binding to ICAM-3 further, solid-phase assays were done using soluble recombinant ICAMs containing the two N-terminal immunoglobulin domains (Fig. 4*b*). THP-1 cells bound to these chimaeric proteins, and binding was blocked by the anti-LFA-1 antibody 38. Thus, the LFA-1 binding site is contained within domains I and II of ICAM-3.

Our results show that the pICAM-3 cDNA encodes a third ligand for LFA-1 and the LFA-1 binding site has been localized to domains I and II. Are the three ICAM ligands for LFA-1 functionally equivalent, or does each one have a unique role? The strict delineation of expression of the ICAMs (ICAM-1 is expressed on activated leukocytes, endothelium and epithelium; ICAM-2 is constitutively expressed on leukocytes and endothelium; ICAM-3 is constitutively expressed only on leukocytes) suggests that each serves a specific purpose rather than acting as a 'failsafe' for the other ICAMs. Furthermore, differences in ligand binding were observed in comparison with ICAMs 1 and 2. ICAM-3 was found to bind LFA-1-expressing cells more weakly than ICAM-1 and ICAM-2. The residues identified as playing a key role in ICAM-1/LFA-1 interactions are conserved in ICAM-3, although other subtle differences may affect interaction with LFA-1. One specific possibility is the presence of *N*-linked carbohydrate groups in domain I of ICAM-3 not found in ICAM-1. The question of whether ICAM-3 binds Mac-1 or p150/95 also remains to be addressed.

In T lymphocytes, engagement of the antigen receptor (TCR)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

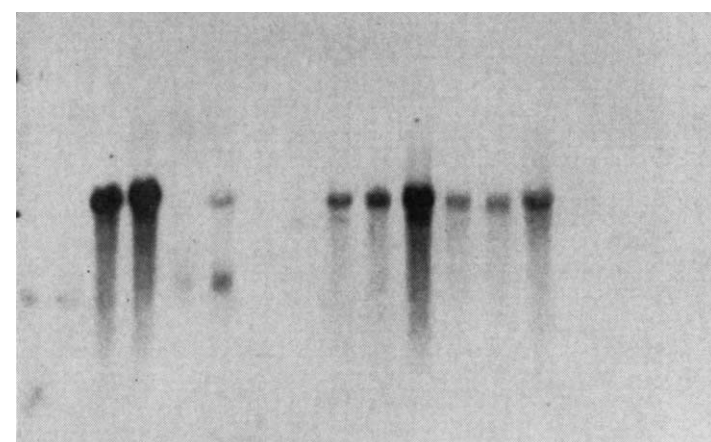
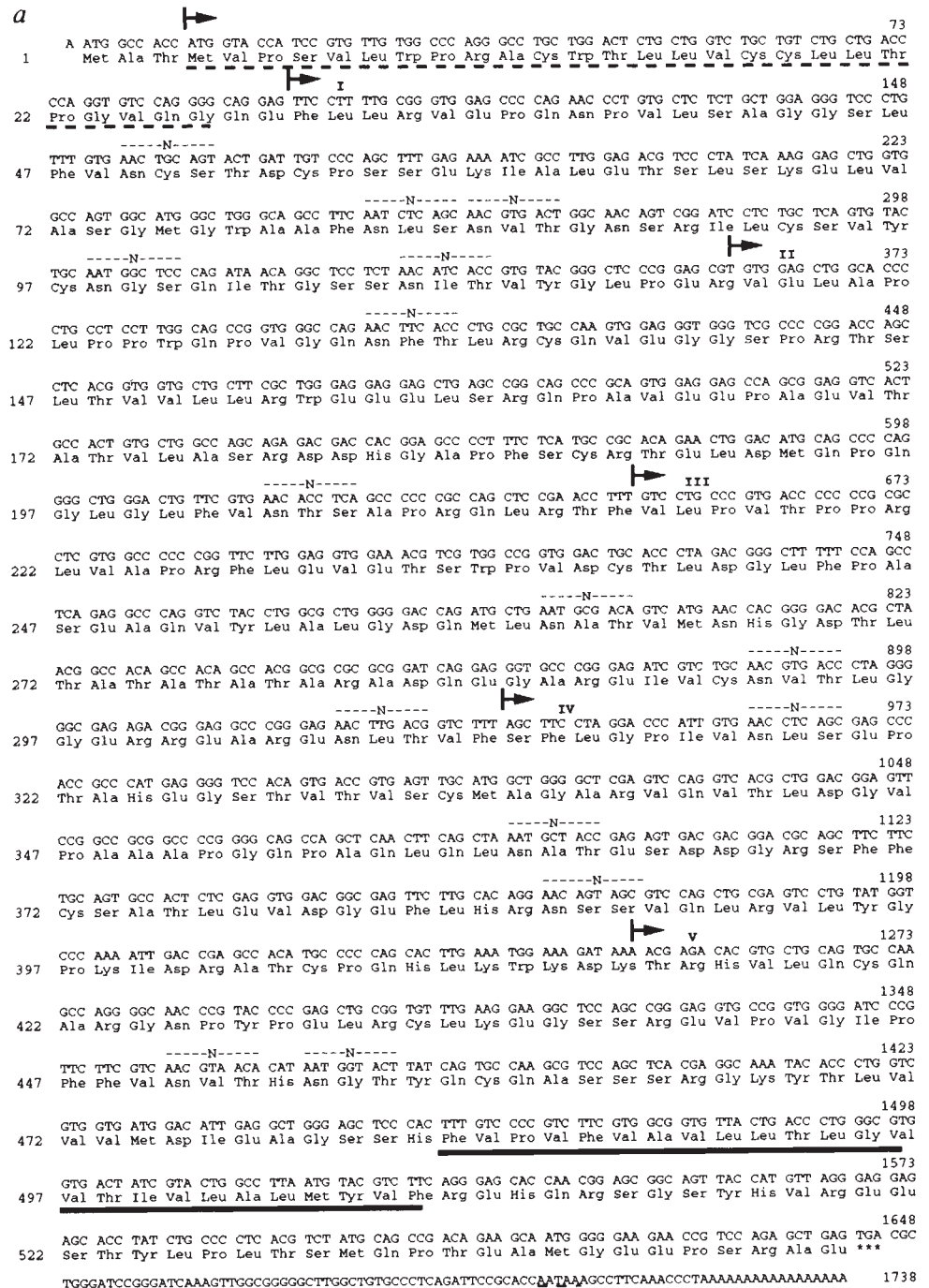


FIG. 2 RNA expression in leukocyte cell lines as determined by RNA blot hybridization. Lane 1, K562; lane 2, TPA induced K562; lane 3, U937; lane 4, TPA induced U937; lane 5, KG1; lane 6, TPA-induced KG1; lane 7, THP-1; lane 8, HL60; lane 9, IM9; lane 10, Ramos; lane 11, Priess; lane 12, Daudi; lane 13, MOLT 4; lane 14, Namalwa; lane 15, Jurkat; lane 16, proliferating human umbilical vein endothelial cells (HUVEC); lane 17, HUVEC stimulated with TNF- α and IL- β ; lane 18, HUVEC grown on Matrigel. Total RNA (10 μ g) was denatured in formaldehyde, electrophoresed, transferred to nylon membranes and hybridized with labelled ICAM-3 cDNA insert; membranes were then washed and autoradiographed. Cells were induced with 10 nM TPA for 24 h. HUVEC were induced with 10 U ml⁻¹ cytokines (British Biotechnology) for 6 h or cultured on Matrigel (Collaborative Research) for 24 h.

FIG. 3 Sequence analysis of ICAM-3. *a*, Nucleotide and deduced amino-acid sequence of pICAM-3 cDNA insert. The sequence on both strands was determined by the chain termination method²³ using a combination of synthetic oligonucleotide primers and sub-clones. The deduced translation is shown beneath the DNA sequence. The putative signal peptide is indicated by a dashed line starting at Met1. The heavy underlined sequence shows a likely transmembrane region. A polyadenylation signal is shown at nucleotide 1,704 (dashed underline). Potential *N*-glycosylation sites are indicated by ---N---. Proposed start points for immunoglobulin domains are indicated above the nucleotide sequence. *b*, Alignment of immunoglobulin domains between ICAM-3 and ICAMs 1 and 2. Alignments were generated using the program PILEUP from the UWGCG²⁴ package with gap-weight and gap-length penalties of 0.5. Domains V of ICAM 1 and 3 were aligned separately.



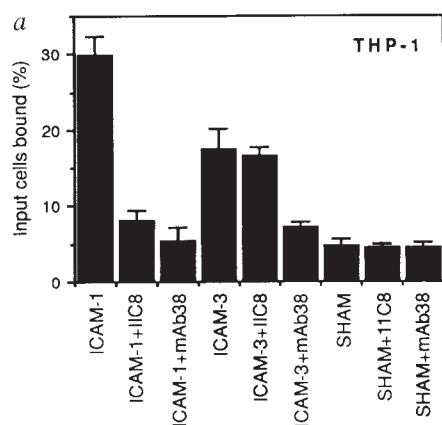
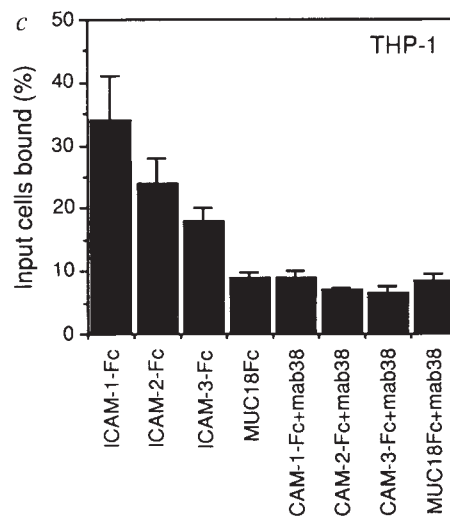
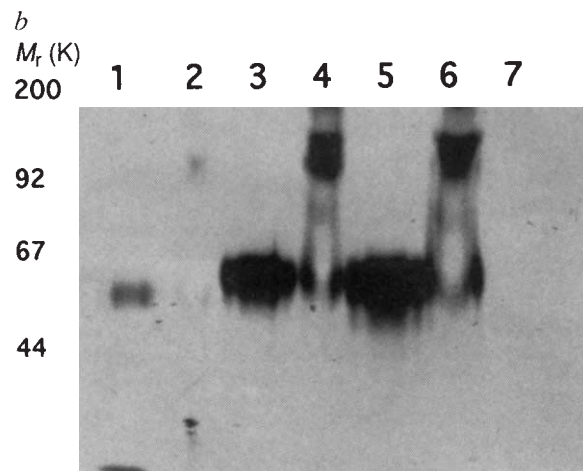


FIG. 4 Functional analysis of ICAM-3/LFA-1 interaction. **a**, COS cells transiently expressing ICAM-1, ICAM-3 or sham transfected, were plated in 96-well assay dishes. ^3H thymidine-labelled THP-1 cells, pretreated with 10 mg ml^{-1} heat-aggregated human IgG, were allowed to adhere for 35 min at 37°C , then ATP added to a final concentration of $20\text{ }\mu\text{M}$. After a further 5 min incubation, dishes were washed three times and bound cells lysed and counted by scintillation assay²⁵. Cells were pretreated for 30 min with blocking antibodies. Monoclonal antibodies (mAb) were: anti-ICAM-1 11C8 ($10\text{ }\mu\text{g ml}^{-1}$ as Fab fragments); anti LFA-1 mAb 38 ($1:100$ as ascites). Results are presented as the means ($n=6$, ± 1 standard deviation) of the per cent of input cells bound. **b**, Preparation of soluble recombinant (sr) ICAMs. Lanes 1 and 2, ICAM-1 Fc; lanes 3 and 4, ICAM-2 Fc; lanes 5 and 6, ICAM-3 Fc; lane 7, vector only control; under reducing (lanes 1, 3, 5, 7) and non-reducing (lanes 2, 4, 6) conditions. SDS-PAGE of ^{35}S -methionine-labelled srICAMs. Recombinant ICAM-IgG1 Fc genes were transiently expressed in COS-1 cells and secreted protein purified from tissue culture supernatants with protein A-coupled Sepharose. The chimaeric genes were created by using polymerase chain reaction (PCR) to amplify two N-terminal domains of each ICAM, with restriction sites compatible for ligation, into an expression vector, pIg1, containing hinge, CH2 and CH3 exons of human IgG₁. A control protein consisting of the whole extracellular domain of MUC18 (ref. 26) fused to IgG₁Fc was also made. PCR conditions were: 94°C for 1 min, 55°C for 30 s and 72°C for 1 min, for 30 cycles. srICAM-1 and 3 and srMUC18 were amplified from cDNA clones and srICAM-2 from a HUVEC cDNA library. PCR primers used were: srICAM-1, 5'-CTAGAGAACCCTGCTTAAC-3' (CDM8FSP) and 5'-GATCGGATCCACTTACCTGTAAGGTCTGGAGCTGGTAGGGGCG-3'; srICAM-2, 5'-GATCAAGCTTTCCCTGCGAGCCGTGGAGACTGC-3' and 5'-GATCGGATCCACTTACCTGTCTGGCTGTCCGACACAGGCTCATA-3'; srICAM-3, CDM8FSP and 5'-GATCAGATCTACTTACCTGTCTGACGGGGGGTACGGGCGAC-3'; srMUC-18 (ref. 26), CDM8FSP and 5'-ACGGATCCACTTACCTGTCCGGCTCTCGGCTCCGGCAGCTT-3'. **c**, Binding of labelled THP-1 cells to wells coated with srICAMs in solid phase. srMUC18 was included as a negative control. Assays were performed in quintuplicate and results presented as



mean per cent of input cells bound, ± 1 standard deviation. 25-well dishes (Sterilin) were treated with 10% toluene in ethanol and washed to remove plasticizers before incubating with $250\text{ }\mu\text{l}$ goat anti-human IgGf at $10\text{ }\mu\text{g ml}^{-1}$ in 50 mM Tris, pH 9.0, for 2–4 h; remaining sites were blocked by incubation with PBS containing bovine serum albumin 2 mg ml^{-1} overnight. srICAMs were bound for 1 h at $10\text{ }\mu\text{g ml}^{-1}$ in PBSA, washed and blocked with heat-aggregated human IgG at 1 mg ml^{-1} . Adhesion assays with labelled THP-1 cells were performed as in **a**.

by antigen leads to rapid but transient upregulation of LFA-1 affinity for ICAM-1 (ref. 20) and this secondary adhesion event is one of the triggers for the lymphocyte-activation cascade. The biological corollary of this is that blocking antibodies against ICAM-1 have been shown effectively to prevent allograft rejection^{21,22}. ICAM-1 expression is only seen, however, in cells that have already undergone some degree of activation and therefore the actual initial adhesion event supporting antigen presentation to the antigen receptor may be between LFA-1 and ICAM-3. Significantly, we have found the highest expression of ICAM-3 messenger RNA in cell types implicated in antigen presentation to T cells, namely B cells and those with monocytic/macrophage phenotype. In these cases, the constitutive expression of ICAM-3 would be pivotally important in the genesis of immune responses. □

Received 10 July; accepted 2 October 1992.

- Dustin, M. L. & Springer, T. A. *Rev. Immun.* **9**, 27–66 (1991).
- Uciechowski, P. & Schmidt, R. E. in *Leucocyte Typing IV. White Cell Differentiation Antigens* (eds Knapp, W.) 543–554 (Oxford Univ. Press, Oxford, 1989).
- Marlin, S. D. & Springer, T. A. *Cell* **51**, 813–819 (1987).
- Simmons, D. L., Makgoba, M. W. & Seed, B. *Nature* **331**, 624–627 (1988).
- Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L. & Springer, T. A. *Cell* **52**, 925–933 (1988).

- Staunton, D. E., Dustin, M. L. & Springer, T. A. *Nature* **339**, 61–64 (1989).
- Moy, V. T. & Brian, A. A. *J. exp. Med.* **175**, 1–7 (1992).
- Altmann, D. M., Hogg, N., Trowsdale, J. & Wilkinson, D. *Nature* **338**, 521–514 (1989).
- Makgoba, M. W. *et al. Eur. J. Immun.* **18**, 637 (1988).
- Needham, L., Pearson, J. D. & Hellewell, P. G. in *Vascular Endothelium: Interactions with Circulating Cells* (eds Gordon, J. L.) 61–89 (Elsevier, Amsterdam, 1991).
- de Fougerolles, A. R., Stacker, S. A., Schwarting, R. & Springer, T. A. *J. exp. Med.* **174**, 253–267 (1991).
- de Fougerolles, A. R. & Springer, T. A. *J. exp. Med.* **175**, 185–190 (1992).
- Knapp, W. *et al. Leucocyte Typing IV. White Cell Differentiation Antigens* (Oxford Univ. Press, Oxford, 1989).
- Seed, B. & Aruffo, A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 8573–8577 (1987).
- Cavener, D. R. & Ray, S. C. *Nucleic Acids Res.* **19**, 3185–3192 (1991).
- von Heijne, G. *Nucleic Acids Res.* **14**, 4683 (1986).
- Staunton, D. E., Dustin, M. L., Erickson, H. P. & Springer, T. A. *Cell* **61**, 243–254 (1990).
- Berendt, A. R. *et al. Cell* **68**, 71–81 (1992).
- Dransfield, I., Cabanas, C., Barrett, J. & Hogg, N. *J. Cell Biol.* **116**, 1527–1535 (1992).
- Dustin, M. L. & Springer, T. A. *Nature* **341**, 619 (1989).
- Cosimi, A. B. *et al. J. Immun.* **144**, 4604–4612 (1990).
- Flavin, T., Rothlein, R., Faanes, R., Ivins, K. & Starnes, V. A. *Transpl. Proc.* **23**, 533–534 (1991).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
- Devereux, J., Haeblerli, P. & Smithies, O. *Nucleic Acids Res.* **12**, 387–395 (1984).
- Simmons, D. L. & Needham, L. in *Vascular Endothelium: Interactions with Circulating Cells* (eds Gordon, J. L.) 1–32 (Elsevier, Amsterdam, 1991).
- Lehmann, J. G., Riehmuller, G. & Johnson, J. P. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9891–9895 (1989).

ACKNOWLEDGEMENTS. J.F. and C.L.L.H. are combined first authors. We thank N. Hogg for anti-LFA-1 antibody, J. McBain for assistance in setting up solid-phase adhesion assays and P. Crocker and A. Drummond for discussion. This work was supported by the Imperial Cancer Research Fund, Leukaemia Research Fund, and British Biotechnology plc.

Cloning and characterization of a new intercellular adhesion molecule ICAM-R

Rosemay Vazeux, Patricia A. Hoffman, Judy K. Tomita, Edna S. Dickinson, Richard L. Jasman, Tom St. John & W. Michael Gallatin*

ICOS Corporation, 22021 20th Avenue SE, Bothell, Washington 98021, USA

THE human intercellular adhesion molecules ICAM-1, ICAM-2 and their counter-receptors, the β_2 or leukointegrins, mediate a variety of homotypic and heterotypic leukocyte and endothelial cell-cell adhesions central to immunocompetence¹. It has been found² that cell-cell adhesion which is dependent on expression of the leukocyte function-associated antigen LFA-1 is not always blocked completely by antibodies raised against ICAM-1 and ICAM-2. Other leukointegrin ligands therefore probably exist, such as a glycoprotein of M_r 124K that binds LFA-1 and has been designated ICAM-3 on the basis of this function³. We have molecularly cloned a new member of the ICAM family, ICAM-R, which is related to ICAM-1 and ICAM-2. The complementary DNA encoding ICAM-R is 1,781 base pairs long and the protein has five extracellular immunoglobulin-family type domains. The mature cell-surface form of the ICAM-R protein has an M_r which varies from 116 to 140K in a cell type-specific fashion. Overall identities in protein sequence with ICAM-1 and ICAM-2 are 48% and 31% respectively, with the degree of similarity varying between individual domains. The high level of expression of ICAM-R on resting leukocytes of all lineages and its lack of expression on either resting or cytokine-activated endothelial cells indicates a pattern of expression distinct from ICAM-1 and ICAM-2. In common with ICAM-1 and ICAM-2, ICAM-R is a ligand for the β_2 -integrin CD11a/LFA-1 (CD18).

We assumed that, as with the genetically defined ICAMs (ICAM-1 and ICAM-2), novel leukointegrin binding partners would be related to the immunoglobulin gene superfamily C2-SET⁴. Therefore, to identify new ICAMs we designed degenerate oligonucleotides based on conserved sequences flanking cysteines that form the characteristic C2-SET intrachain disulphide bonds. Using these primers, polymerase chain reaction products amplified from human genomic DNA were subcloned and sequenced (Fig. 1 legend). One clone, 13-3C7, contained a 170-base-pair (bp) insert with a predicted protein sequence 77% identical to domain II of ICAM-1, and 31% identical to domain II of ICAM-2. A cDNA library derived from the human myelomonocytic cell line, HL-60, was then screened by hybridization with the 13-3C7 insert to isolate the corresponding complete cDNA. The full-length clone isolated encoded a novel protein we designated 'ICAM-R' because of its relatedness to ICAM-1 and ICAM-2 and its affinity for LFA-1.

The ICAM-R cDNA (Fig. 1a) is 1,781 base pairs (bp) long, with a 1,640-bp open reading frame starting with an ATG at position 16, followed by a second ATG at position 24, which is more typical of a consensus initiation sequence⁵. The open reading frame is followed by 82 bp of 3' untranslated region, a polyadenylation signal at position 1,718 and a poly(A) tail beginning at position 1,738. The predicted protein has a 29-amino-acid putative signal sequence⁶ followed by 518 amino acids of the mature protein, with an expected core polypeptide M_r of about 56K. A putative 24-amino-acid hydrophobic transmembrane domain is followed by a 37-amino-acid potential cytoplasmic region. ICAM-R contains 15 potential N-linked glycosylation sites. Like ICAM-1, the extracellular portion contains five domains with intrachain disulphide bonds typical of

TABLE 1 FACS analysis of human cell lines, resting peripheral blood leukocytes and human endothelial cells using monoclonal antibodies against ICAM-R (26H11C), ICAM-1 and ICAM-2

		ICAM-R	ICAM-1	ICAM-2
T cell	CEM	109/99*	11/46	83/99
T cell	SUP-T1	30/96	15/92	140/99
T cell	SKW-3	178/99	14/85	90/99
B cell	JUJOYE	46/98	301/99	21/98
B cell	RAJI	28/96	90/99	33/99
Monocytic	HL-60	60/99	11/74	25/98
Monocytic	U937	42/99	60/99	20/99
Myeloid	KG-1	291/99	50/99	56/99
Erythroleukaemic	K562	5/10	43/99	48/99
Endothelial cells	HuVe	6/0.5	18/18	ND
Endothelial cells	HuVe-TNF	5/0.5	278/99	ND
	Lymphocytes	160/97	13/25	43/91
	Monocytes	267/93	69/91	128/98
	Granulocytes	128/83	21/57	12/3

Cell lineages are indicated in the left column. Both the mean channel fluorescence intensity and the percentage of positive cells relative to a negative control are indicated. In parallel experiments the other ICAM-R antibodies showed similar reactivity on these different cells. Stainings were carried out as described in Fig. 2 legend, using either anti-ICAM-R mAb supernatant (26H11C) or anti-ICAM-1 and anti-ICAM-2 mAbs. HUVE endothelial cells were cultured for two passages, then were either unstimulated or stimulated with TNF- α (2 ng ml⁻¹ for 18 h) before staining. To stain resting peripheral blood leukocytes, monocytes and lymphocytes were purified by Ficoll-PAQUE gradients (Pharmacia) and the granulocytes were recovered from the pellet after lysis of the erythrocytes with ammonium chloride. Cells were then stained by incubation with the appropriate mAbs and each cell population was identified by FACS analysis. ND, Not done.

* Mean fluorescence/per cent positive cells.

immunoglobulin-like loops (Fig. 1b). Overall protein sequence identity of ICAM-R is greater with ICAM-1 (48%) than with ICAM-2 (31%), but identity between ICAM-R and ICAM-1 varies markedly between individual domains (Fig. 1b). For example, the N-terminal domains of ICAM-R and ICAM-1 show limited identity (37%), whereas domain II is quite conserved (77%). Significantly, despite overall divergence in the first domains, two key residues, E34 and Q73, essential for ICAM-1 binding to LFA-1 (ref. 7), are preserved in all three molecules. Therefore, these amino acids may either confer specificity for LFA-1 binding or be essential to a general β_2 -integrin binding conformation.

A single RNA transcript of ~2.2 kilobases (kb) was detected in all the principal leukocyte lineages (Fig. 2a). In contrast to ICAM-1, ICAM-R messenger RNA was not expressed by cultured endothelial cells even after stimulation with tumour necrosis factor (TNF- α). For further comparison, we subcloned ICAM-R and ICAM-1 cDNAs into the expression vector pcDNAIneo (Invitrogen), stably transfected them into mouse L-cells and used them to screen for ICAM-R-specific monoclonal antibodies after immunizing mice with HL-60 cells. Four such antibodies were selected (Fig. 2 legend). All reacted specifically with ICAM-R but not with ICAM-1 transfectants (Fig. 3a).

Using these reagents, cell-surface expression of ICAM-R was detected on normal leukocytes and transformed cell lines of all lineages except the erythroleukaemic line K562 (Table 1). ICAM-1, ICAM-2 and ICAM-R were usually co-expressed, but the high levels of ICAM-R on normal resting leukocytes were in contrast with the low ICAM-1 expression seen in the absence of activating stimuli. Also, ICAM-R was strongly expressed on granulocytes but ICAM-2 was undetected on these cells. Moreover, unlike ICAM-1 and ICAM-2, ICAM-R was undetected on resting or cytokine-stimulated cultured endothelial cells. Widespread ICAM-R distribution on leukocytes *in vivo* was confirmed by immunohistological staining of human lymph

* To whom correspondence should be addressed.

a cagctctctgtcaga ATG GCC ACC ATG GTA CCA CTC TGG TTG TGG (45)
M A T N V P S V L W -20

CCC AGG GCC TGC TGG ACT CTG CTG GTC TGT TGT CTG ACC CCA (90)
P R A C W T L L V C C L L T T P -5

GGT GTC CAG GGC CAG GAG TTC CTT TTG CGG GTG GAG GCC CAG AAC (135)
G V Q G Q E F L L R V E P Q N

CCT GTG CTC TCT GCT GGA GGG TCC CTG TTT GTG AAC TGC AGT ACT (180)
P V L S A G G S L F V N S T 26

GAT TGT CCC AGC TCT GAG AAA ATC GCC TTG GAG AGC TCC CTA TCA (225)
D C P S S E K I A L E P T S L S 41

AAG GAG CTG GTG GCC AGT GGC ATG GGC TGG GCA GCC TTC AAT CTC (270)
K E L V A S G M G W A A F N L 56

AGC AAC GTG ACT GGC AAC AGT CGG ATC CTC TGC TCA GTG TAC TGC (315)
S N V T G N S R I L C S V Y C 71

AAT GGC TCC CAG ATA ACA GGC TCC TCT AAC ATC ACC GTG TAC GGG (360)
N G S Q I T G S S N I T V Y G 86

CTC CCG GAG CGT GTG GAG CTG GCA CCC CTG CCT CTG TGG CAG CCG (405)
L P E R V E L A L E P T W Q P 101

GTG GGC CAG AAC TTC ACC CTG CGC TGC CAA GTG GAG GGT GGG TCG (450)
V G Q N F T L R C Q V E G G S 116

CCC CGG ACC AGC CTC AGT GTG GTG CTG CTT CGC TGG GAG GAG GAG (495)
P R T S L T T V V L L R W E E E 131

CTG AGC CGG CAG CCC GCA GTG GAG GAG CCA GCG GAG GTC ACT GCC (540)
L S R Q P A V E E P A E V T A 146

ACT GTG CTG GCC AGC AGA GAC GAC CCA GGC CCT TTC TCA TGC (585)
T V L A S R D D H G A P F S C 161

CGC ACA GAA CTG GAC ATG CAG CCC CAG GGC GTG GGA CTG TTC GTG (630)
R T E L D M Q P Q G L G L F V 176

AAC ACC TCA GCC CCC CAG CTG CCA AAT TTT GTG CTG CCC GTG (675)
N T S A P R Q L R T F V L P V 191

ACC CCC CGG CGC CTC GTG GCC CCC CGG TTC GTG GAG GAA ACG (720)
T P P R L V A P R F L E V E T 206

TGG TGG CCG GTG GAC TGC ACC CTA GAG GGC CTT TTT CCA ACC TCA (765)
S W P V D C T L D G L F P A S 221

GAG CGC CAG GTC TAC CTG GCG GGT GCG CAG ATG CTG AAT GCG (810)
E A Q V Y L A L G D Q M L N A 236

ACA GTC ATG AAC CAC GGC GAC ACG CTA ACG GCC ACA GCC ACA GCC (855)
T V M N H G D T T L T A T A T A 251

ACG CGC CGC GCG GAT CAG GAG GGT GCG GCG GAG ATC GTC TGC AAC (900)
T A R A D Q E G A R E I V C N 266

GTG ACC CTA GGC GGC GAG AGA CCG GAG GCC CGG GAG AAC TTG ACG (945)
V T L G G E R R E A R E N L T 281

GTG TTT AGC TTT CTA GCA CCC ATT GTG AAC CTC AGC GAG ACC CCG (990)
V F S F L G P I V N S E P T 296

GCC CAT GAG GGC TCC ACA GTG ACC GTG AGT GTC ATG GCT GGG GCT (1035)
A H E G S T V T V S C M A G A 311

CGA GTC CAG GTC ACG CTG GAC GGA GTT CCG GCC GCG GCC CCG GGG (1080)
R V Q V T L D G V P A A A C P G 326

CAG CCA GCT CAA CTT CAG CTA AAT GCT ACC GAG AGT GAC GAC GGA (1125)
Q P A Q L Q L N A T E S D D G 341

CGC AGC TTT TGC AGT GCC ACT CTG GAG GTG GAC GGC GAG TTC (1170)
R S F F C S T L E V G D G E F 356

TTG CAC AGG AAC AGT AGC GTC CAG CTG CAG GTC CTG TAT GGT CCC (1215)
L H R N S S V Q L R V L Y G P 371

AAA ATT GAC CGA GCC ACA TGC CCC CAG CAC TTG AAA TGG AAA GAT (1260)
K I D R A T C P C G H L K W A D 386

ACG AGA CAC GTC CTG CAG TGC CAA GCC AGG GGC AAC CCG TAC (1305)
K T R H V L Q C Q A R G N P Y 401

CCC GAG CTG CGG TGT TTG AAG GAA GGC TCC AGC CGG GAG GTC CCG (1350)
P E L R C L K E G V S A A E V P 416

GTG GGC ATC CCG TTC TTC GTC AAC GTA ACA CAT AAT GGT ACT TAT (1395)
V G I P F F V N V T H N G T Y 431

CAG TGC CAA GGC TCC AAG TCA CGA GGC AAA TAC ACC CTG GTC GTG (1440)
Q C Q A S S R S G K Y T L V V 446

GTG ATG GAC ATT GAG GCT GGC AGC TCC CAC TTT GTC CCC GTC TTC (1485)
V M D I E A G S S H F V P V F 461

GTG GCG GTG TTA CTG ACC CTG GGC GTG GTG ACT ATC GTA CTG GCC (1530)
V A L L L L G V V T I V L A 476

TTA ATG TAC GTC TTC AGG GAG CAC CAA CGG AGG GGC AGT TAC CAT (1575)
L N Y V F R E H Q R S G S Y H 491

GTT AGG GAG GAG AGC ACT TAT CTG CCC CTC AGC TCT ATG CAG CCG (1620)
V R E E S T Y L P L T S M Q P 506

ACA GAA GCA ATG GGG GAA GAA CCG TCC AGA GCT GAG tgacgctggg (1667)
T E A M G E E P S R A E 518

tccgggataaagtggcggggcttggtgtgctccctcagattccgcacccaataaagcc (1726)
ttcaactcccccaaaagaaagaaagaaagaaagaaagaaagaaagaaagaaagaa (1781)

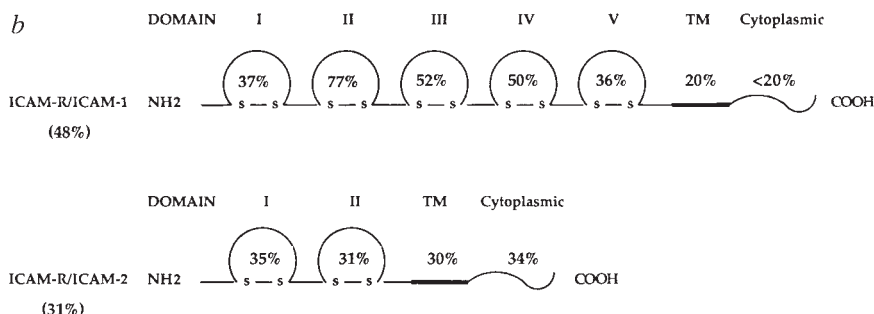


FIG. 1 Sequence of ICAM-R. *a*, Complete nucleotide sequence of ICAM-R and predicted protein sequence. Nucleotide sequence is labelled on the right in parenthesis. Amino acids are numbered at right from the first residue after the predicted signal peptide cleavage site. The potential polyadenylation signal is underlined, the potential signal peptide and transmembrane domain are shaded, and potential *N*-glycosylation sites are delineated with an arrow and the limits of the five external immunoglobulin-like domains are indicated by vertical lines. *b*, Comparison between the amino-acid sequences of ICAM-R with ICAM-1 and ICAM-2. Identities between the overall mature proteins (in parenthesis) and between their domains are represented. The last three domains of ICAM-R were not used in the alignment with the ICAM-2 mature protein. TM, transmembrane.

METHODS. Degenerate oligonucleotides related to the C2-SET of adhesion molecules were selected from amino-acid sequences of ICAM-1, ICAM-2, human vascular adhesion molecule (VCAM-1) and of their closest related genes: mouse neural cell-adhesion molecule, human myelin-associated glycoprotein, human fibroblast growth factor receptor and human platelet endothelial cell-adhesion molecule (PECAM-1). Because each domain of these molecules may have evolved independently^{4,14}, each CAM sequence was subdivided into individual immunoglobulin-like domains, pooled and aligned using the MULTALIN program¹⁵. The second domains of ICAM-1, ICAM-2 and VCAM were more closely related to each other than to other domains of the same or other molecules. We used their consensus amino-acid motifs surrounding the cysteines that form the intrachain disulphide bond of each domain to design the primers. Thus, KNLTLCRC and KSFTIEC motifs were used to design the 5' degenerate oligonucleotides: 5'-ATTCTGCAGGCAAG(AA)(CT)(GC)(AC)(CA)(T)(GCT)(CA)(G)(CG)TG-3', 5'-ATTCTGCAGGCAAG(AA)(CT)(CT)(AC)(CA)(T)(GCT)(CA)(G)(CG)TG-3' and 5'-ATTCTGCAGGCAAG(AA)(CT)(CT)(CT)(CT)(AC)(CA)(T)(GCT)(CA)(G)(CG)TG-3'. The consensus amino-acid motifs DHHGANF and EDGHRNF were used to design the 3' degenerate oligonucleotides: 5'-ATTCTAGAG(AA)(GA)TT(GA)GC-(GC)(CC)(GA)TG(GA)T(GC)(GA)TC-3' and 5'-ATTCTAGAG(AA)(GA)TT(GC)(GT)(GA)TG(GC)(CC)(GA)T-(GC)(GT)TC-3'. *Pst*I and *Xba*I sites were added respectively in the 5' and 3' primers (underlined). The 5' and 3' primers were mixed to a final concentration of 10 µg ml⁻¹ in a polymerase chain reaction (PCR) to amplify human genomic DNA. PCR was done in 2 mM MgCl₂, 25 mM KCl, 10 mM Tris, pH 8.3, 200 mM deoxynucleotides and *Taq* polymerase. After denaturation at 94 °C for 4 min, 35 cycles were run with annealing at 60 °C for 2 min, elongation at 72 °C for 4 min and denaturation at 94 °C for 1 min. A DNA band migrating at about 0.2 kb (the size of an immunoglobulin-like domain) was purified, digested by *Xba*I and *Pst*I, cloned into a phagemid vector and transformed into XL1-blue cells. Single-strand templates were obtained and their sequence determined to obtain an ICAM-2 probe. For larger scale experiments, colonies were first screened for ICAM-1 and ICAM-2 sequences as follows. We synthesized single-strand templates in 96-well plates by growing individual colonies in 300 µl of L-broth containing M13K07 helper phage, carbenicillin and kanamycin. 10 µl each template were transferred with a 96-prong device to a nylon membrane, denatured and fixed with ultraviolet light. Oligonucleotide probes corresponding to ICAM-2 and ICAM-1 domain II were phosphorylated using [γ-³²P]ATP. The nylon membranes were then hybridized in 20% formamide, 5 × SSC, 5 × Denhardt's solution, 0.5% SDS at 42 °C, then washed in 0.2 × SSC three times at room temperature and once at 37 °C for 15 min each. Roughly half of the fragments hybridized with either ICAM-2 or ICAM-1. We determined the sequence of the inserts from the non-hybridizing wells. One of these, clone 13-3C7, was highly homologous to domain II of ICAM-1. We used this clone to screen an HL-60 cell line cDNA library subcloned in phage λSJ34521 (ref. 16). Seven clones ranging from 0.7 to 1.78 kb hybridized with the 13-3C7 probe but not with an ICAM-1 probe. The sequence of these clones was determined. The longest one, PVZ147, fulfilled all criteria to be a full-length cDNA sequence.

FIG. 2 Expression of ICAM-R mRNA and glycoproteins. *a*, Northern blots of 10 μ g total mRNA purified from JJOYE, SKW-3, RAJI cells and from human umbilical vein endothelial cells (HUVE) either stimulated by TNF- α (2 ng ml $^{-1}$ for 18 h), by lipopolysaccharide (LPS; 5 μ g ml $^{-1}$ for 6 h) or unstimulated. The position of the size markers are indicated in kilobases. *b*, Immunoprecipitation of biotinylated ICAM-R glycoprotein extracted from SKW-3, KG-1, HL-60, U937 with monoclonal antibody 26H11C (lane A) or a control mAb (lane B). This western blot shows the four different sizes of the ICAM-R molecule detected on the different cell lines. Pre-stained markers (Biorad) on the right show the apparent molecular weight of the proteins (+/- 10% kD).

METHODS. Northern blot: total RNA samples were fractionated on a 1% agarose gel containing 0.66M formaldehyde, transferred to a nylon membranes and fixed with ultraviolet light. ICAM-1 and ICAM-R DNA probes were labelled with 32 P either by random priming or by PCR amplification. Membranes were hybridized in 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE, 1% SDS at 42 $^{\circ}$ C then washed in 2 $\times$ SSPE/0.1% SDS at room temperature and in 0.5 $\times$ SSPE/0.1% SDS at 50 $^{\circ}$ C for 10 min. Production of mAbs and immunoprecipitations: 6–12-week-old Balb/c mice (Charles River Biotechnical) were immunized with 6 $\times 10^6$ HL-60 cells every 3 weeks. The spleen was fused with NS-1 myeloma cells after the third injection and hybridomas obtained according to standard procedure. Culture supernatants were analysed by FACS on untransfected L-cells, L-cells transfected with ICAM-R cDNA and ICAM-1 cDNA. Anti-ICAM-R antibodies were obtained from four hybridoma cell lines and were isotypized in an ELISA assay. We obtained clones 26E3D (IgG2a), 26H11C (IgG1), 26I10E (IgG1), 26I8F (IgG1). Cell-surface proteins of human leukocyte cells were labelled using sulfo-NHS-biotin (Pierce Chemical). After washes, 0.5–1 $\times 10^7$ cells were incubated in 1 mM sulfo-NHS-biotin in PBS for 5 min at 37 $^{\circ}$ C then washed twice with PBS. Cells were pelleted and solubilized in 10 mM Tris, pH 8, 50 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA. The soluble material was sequentially incubated with normal mouse serum and with protein A-Sepharose beads (Sigma) coupled to rabbit anti-mouse immunoglobulin (Zymed) respectively for 1 h and 30 min at 4 $^{\circ}$ C. Sepharose beads were removed by centrifugation. Specific immunoprecipitation was then performed by incubating the samples with protein A-Sepharose beads prearmed with rabbit anti-mouse immunoglobulin coupled either with 26H11C mAb or with an irrelevant IgG1 mAb and blocked with BSA. Following overnight incubation at 4 $^{\circ}$ C, Sepharose beads were pelleted, washed 4 times with 10 mM HEPES, pH 7.3, 150 mM NaCl, 1% Triton X-100. Samples were boiled for 5 minutes in 50 μ l 50 mM Tris, pH 6.8, bromophenol blue, 20 mM iodoacetamide, 1% SDS and 20% glycerol. After centrifugation the samples (35 μ l) were loaded on a SDS-PAGE gel (8% acrylamide), electroblotted onto Immobilon-P membranes (Millipore) and incubated overnight at 4 $^{\circ}$ C in 5% bovine serum albumin, Tris-buffered saline, 0.2% Tween-20. Western blots were then incubated with streptavidin coupled to horseradish peroxidase (Vector), rinsed, stained using ECL western blotting detection reagents (Amersham) and visualized by chemiluminescence.

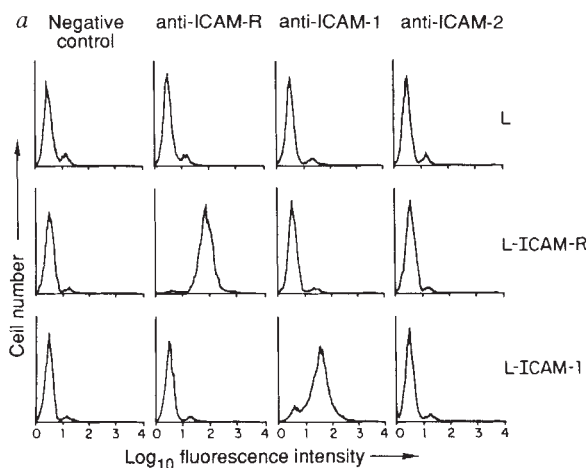
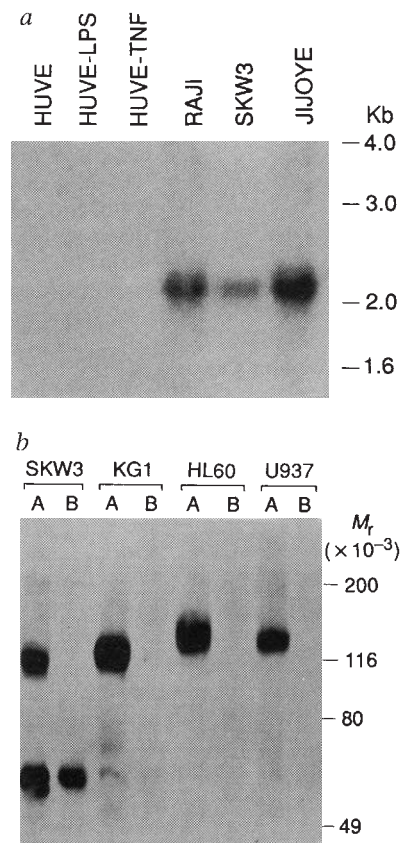
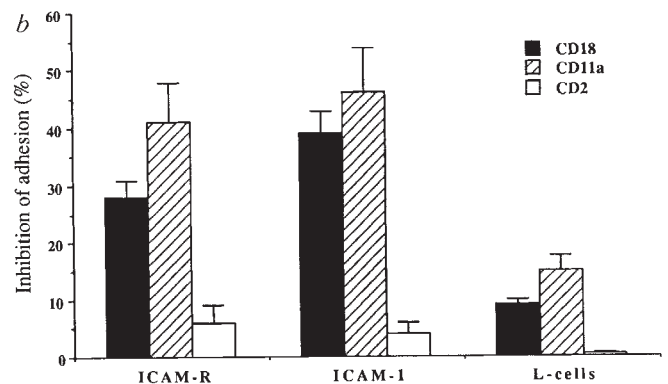


FIG. 3 Expression of ICAM-1 and ICAM-R on L-cell transfectants and adhesion assay. *a*, FACS analysis of ICAM-1, ICAM-R transfectants and control L-cells, using mAbs anti-ICAM-R (26H11C), anti-ICAM-1 (LB2; ref. 17) and anti-ICAM-2 (CBR-IC2/2; Biosource International). Similar staining was obtained with the other anti-ICAM-R mAbs. *b*, Antibody inhibition of adhesion of SKW-3 cells to ICAM-R transfectants, ICAM-1 transfectants or untransfected L-cells. **METHODS.** FACS analysis: 1.5 $\times 10^5$ cells were incubated on ice in 96-well plates with either anti-ICAM-R mAb supernatant (100 μ l) or with purified anti-ICAM-1 and anti-ICAM-2 (1 μ g ml $^{-1}$), then washed. Cells were stained with sheep F(ab') $_2$ sheep anti-mouse anti-IgG fluorescein isothiocyanate-conjugated (Sigma), washed, fixed in 1% paraformaldehyde and analysed with a FACScan (Becton-Dickinson). Staining with 26H11C mAb is shown. Adhesion assay: untransfected L-cell or L-cells transfected either with ICAM-R (L-ICAM-R) or ICAM-1 (L-ICAM-1) were seeded in 24-well tissue culture plates (3 $\times 10^5$ cells per well) 24–48 h before the assay. SKW-3



cells were washed in serum-free RPMI and labelled with Calcein-AM (Molecular Probes, Oregon). Half of these cells were stimulated with phorbol myristylacetate (PMA; 10 ng ml $^{-1}$) for 20 min at 37 $^{\circ}$ C. Both unstimulated and stimulated calcein labelled SKW-3 cells (5 $\times 10^5$ per well) were added to confluent monolayers of adherent cells (L-cells, L-ICAM-R, L-ICAM-1) in 24-well plates and incubated for 30 min at 37 $^{\circ}$ C in RPMI, 1% fetal calf serum (FCS). In mAb blocking experiments, SKW-3 cells were pre-treated with anti-CD18 (TS1/18, ATCC) or anti-CD11a (TS1/22, ATCC) supernatant or anti-CD2 purified mAb (1 μ g ml $^{-1}$) for 30 min at room temperature before incubation with the adherent cell monolayers. Unbound cells were aspirated and wells were filled with RPMI-FCS. Plates were then sealed, centrifuged in an inverted position at 200 r.p.m. for 4 min and aspirated. This centrifugation was followed by a last wash with RPMI-FCS. Plates were then scanned with an automatic fluorescence reader (Cytofluor 2300–2350, Millipore). Results are expressed as per cent inhibition by the mAbs of the adhesion observed with stimulated SKW-3 cells ($\pm$ s.d.) on the adherent cell monolayers. On the vertical axis the percentage of inhibition is represented, on the horizontal axis the cell lines tested are shown and the columns correspond to the different mAbs.

primary sequence, together with the many potential *N* and *O*-linked glycosylation sites, implies heavy post-translational modification of the mature cell-surface forms.

We demonstrated directly that ICAM-R is a β_2 -integrin ligand by adhesion of SKW-3 cells to ICAM-R transfectants. SKW-3 cells were pretreated with phorbol ester to activate LFA-1-dependent adhesion⁸ and assayed for binding to untransfected L-cells and to ICAM-1 and ICAM-R transfectants. Adhesion of stimulated SKW-3 cells to either transfectant was inhibited by monoclonal antibodies against either α (CD11a) or β (CD18) chains of LFA-1 but was unaffected by a control anti-CD2 reagent (Fig. 3b). Although none of the four α ICAM-R antibodies blocked adhesion to L-cell transfectants, these antibodies modulate other cell contact-dependent immune events such as T-cell proliferation (manuscript in preparation).

There is evidence, therefore, that all three genetically defined ICAMs, ICAM-1, ICAM-2 and ICAM-R bind LFA-1. As the properties of ICAM-R and ICAM-3 (ref. 3) seem to be similar, it will be interesting to determine the homology between them once the sequence of ICAM-3 is established.

Two obvious differences between ICAM-R and ICAMs 1 and 2 are the lack of expression of ICAM-R on endothelial cells and the abundance of ICAM-R on resting leukocytes. One role of ICAM-R might, therefore, be in those leukocyte-leukocyte interactions that precede the transcriptional upregulation of ICAM-1 which occurs upon cell activation⁹. Given the distinctive character of the ICAM-R cytoplasmic domain (Fig. 1b) ICAM-R-specific adhesive or signal transduction activities might also be fostered by unique associations with cytoplasmic or cytoskeletal elements. Sequence divergence in the putative integrin-binding, N-terminal domain of ICAM-R suggests the possibility that it also interacts with counter-receptors in addition to the CD11a/CD18 complex. The demonstration that a variety of immune cell-cell interactions (mixed lymphocyte reaction¹⁰, cytotoxicity¹¹, natural killer cell activity¹², leukocyte-epithelium adhesion¹³) requiring CD18/CD11a can occur independently of ICAM-1 recognition underscores the need to dissect the respective roles of the other ICAMs in these functions and to determine whether additional members of the ICAM subgroup of glycoproteins exist. □

Received 17 July; accepted 2 October 1992.

1. Springer, T. A. *Nature* **346**, 425–434 (1990).
2. de Fougerolles, A. R., Stacker, S. A., Schwarting, R. & Springer, T. A. *J. exp. Med.* **174**, 253–267 (1991).
3. de Fougerolles, A. R. & Springer, T. A. *J. exp. Med.* **175**, 185–190 (1992).
4. Williams, A. F. & Barclay, N. A. *Rev. Immun.* **6**, 381–405 (1988).
5. Kozak, M. *Nucleic Acids Res.* **15**, 8125–8148 (1987).
6. von Heijne, G. *Nucleic Acids Res.* **14**, 4683–4690 (1986).
7. Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L. & Springer, T. A. *Cell* **52**, 925–933 (1988).
8. Dustin, M. L. & Springer, T. A. *Nature* **341**, 619–624 (1989).
9. Pober, J. S. et al. *J. Immun.* **137**, 1893–1896 (1986).
10. Bagnasco, M., Pesce, G., Pronzato, C. & Canonica, G. W. *Cell Immun.* **128**, 362–369 (1990).

11. Makgoba, M. W. et al. *Eur. J. Immun.* **18**, 637–640 (1988).
12. Akella, R. & Hall, R. E. *Eur. J. Immun.* **22**, 1069–1074 (1992).
13. Tosi, M. F. et al. *Am. J. Respir. Cell Molec. Biol.* **7**, 214–221 (1992).
14. Hunkapiller, T. & Hood, L. *Adv. Immun.* **44**, 1–63 (1989).
15. Corpet, F. *Nucleic Acids Res.* **16**, 10881–10890 (1988).
16. Nottenburg, C., Gallatin, W. M. & St John, T. *Gene* **95**, 279–284 (1990).
17. Clark, E. A., Ledbetter, J. A., Holly, R. C., Dinndorf, P. A. & Shu, G. *Hum. Immun.* **16**, 100–113 (1986).

ACKNOWLEDGEMENTS. We thank M. Czajkowski and L. Muller for FACS stainings, L. Wilburn for immunohistological analysis, A. Hoffstetter for preparation of the manuscript, M. Emerman, G. Peterman and P. Kilgannon for reviewing the manuscript, E. Clark for LB-2 Mab, L. Graf for the HL-60 cDNA library, and S. Aruffo for the ICAM-1 cDNA.

Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in *E. coli*

Philippe Bouvet*† & Joel G. Belasco*‡

* Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

† Laboratoire de Biologie et Genetique du Developpement, UA CNRS 256, Université de Rennes I, 35042 Rennes Cedex, France

DESPITE the variety of messenger RNA half-lives in bacteria (0.5–30 min in *Escherichia coli*) and their importance in controlling gene expression, their molecular basis remains obscure. The lifetime of an entire mRNA molecule can be determined by features near its 5' end, but no 5' exoribonuclease has been identified in any prokaryotic organism^{1–6}. A mutation that inactivates *E. coli* RNase E also increases the average lifetime of bulk *E. coli* mRNA and of many individual messages, suggesting that cleavage by this endonuclease may be the rate-determining step in the degradation of most mRNAs in *E. coli*^{7–16}. We have investigated the substrate preference of RNase E in *E. coli* by using variants of RNA I, a small untranslated RNA whose swift degradation *in vivo* is initiated by RNase E cleavage at an internal site. We report here that RNase E has an unprecedented substrate specificity for an endoribonuclease, as it preferentially cleaves RNAs that have several unpaired nucleotides at the 5' end. The sensitivity of RNase E to 5'-terminal base pairing may explain how determinants near the 5' end can control rates of mRNA decay in bacteria.

An advance in understanding the structural basis for differential mRNA stability in bacteria was the recent discovery that a 5'-terminal stem-loop enables the *E. coli ompA* 5' untranslated region to prolong the *in vivo* lifetime of most heterologous

messages to which it is fused (refs 17–19; M. Hansen and J.G.B., manuscript in preparation). The sequence of this stem-loop is relatively unimportant, but its location very near the 5' end is crucial, as the presence of just five unpaired nucleotides (nt) upstream of this hairpin can completely negate its contribution to mRNA stability. These and other findings suggest that *E. coli* contains an important ribonuclease that preferentially degrades messages beginning with several unpaired nucleotides¹⁹. As preliminary evidence indicated that this enzyme is not a 5' exonuclease that removes single nucleotides sequentially from the mRNA 5' end¹⁹, we decided to test whether a 5'-terminal stem-loop imposes a barrier to internal cleavage of RNA by RNase E. We therefore examined the degradation of RNA I from plasmid pBR322 (Fig. 1), a 108-nt RNA that controls plasmid copy number^{20–23}. In *E. coli*, rapid decay of RNA I is initiated by RNase E cleavage at a unique site 5 nt from the 5' end^{24,25}. This cleavage occurs with a half-time of 2–3 min and triggers swift degradation of the resulting RNA I fragments²⁵.

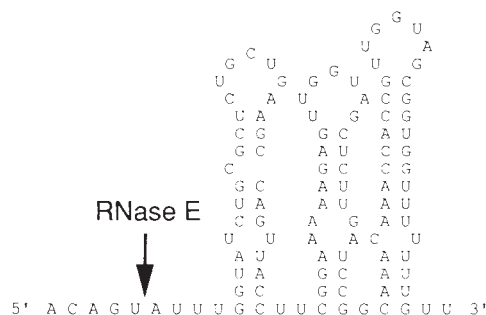


FIG. 1 Secondary structure of pBR322 RNA I. The indicated structure is as reported previously^{22,23}. The site of endonucleolytic cleavage by RNase E²⁴ is marked with an arrow.

† To whom correspondence should be addressed.

We first added a simple stem-loop structure (hp*: GAUCG-CACCGGCAGCUGCCGGUGGGCGAUC) to the 5' end of RNA I to generate RNA I.10. A plasmid encoding both RNA I.10 and wild-type RNA I was introduced into *E. coli* strain N3433, and degradation of these RNAs was monitored after inhibition of transcription (Fig. 2a). This simple modification stabilizes RNA I fivefold, increasing its half-life to 13 min (Table 1). Two other 5'-terminal hairpins were also tested: hpX (GAUCCUCGAGGGGAUC) in RNA I.20 and *ompA* hp1¹⁹ in RNA I.30. In each case the lifetime of RNA I is significantly prolonged, though less markedly in the case of RNA I.30 (Table 1).

To determine whether the protective effect of these stem-loops was due to their location at the 5' end of the RNA or their proximity to the site of RNase E cleavage, the distance between the 5' stem-loop and either the internal cleavage site or the 5' end was increased. Adding four nucleotides (GAUC) immediately downstream of hp*, to generate RNA I.40, nearly doubled

the distance between the 5' stem-loop and the RNase E cleavage site but had no effect on the ability of the 5' hairpin to stabilize RNA I. RNA I.40 has a half-life equal to that of RNA I.10 and five times longer than that of another RNA I variant (RNA I.4) bearing the same four additional nucleotides but lacking a 5' stem-loop (Table 1). In contrast, adding just five unpaired nucleotides (GAUCC or GAUCA) upstream of the 5'-terminal stem-loop of RNA I.10 or RNA I.20 completely eliminates the stabilizing influence of the added hairpin (RNA I.15 and RNA I.25; Fig. 2b and Table 1). Alkylation of unpaired bases in RNA I.20 and RNA I.25 with dimethylsulphate (DMS) or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate (CMCT) confirmed that the secondary structures of these two RNAs are identical apart from the five additional unpaired nucleotides at the 5' end of RNA I.25 (Fig. 3a). These data indicate that the ability of a 5' stem-loop to stabilize RNA I is due to the resulting absence of unpaired nucleotides at the 5' end.

As observed for wild-type RNA I²⁵, degradation of RNA I.15 and RNA I.25 is much slower when RNase E is inactivated in an isogenic *E. coli* strain (N3431) with a temperature-sensitive

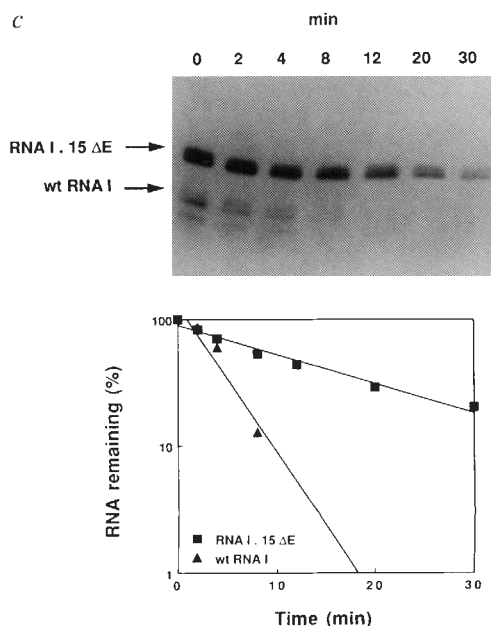
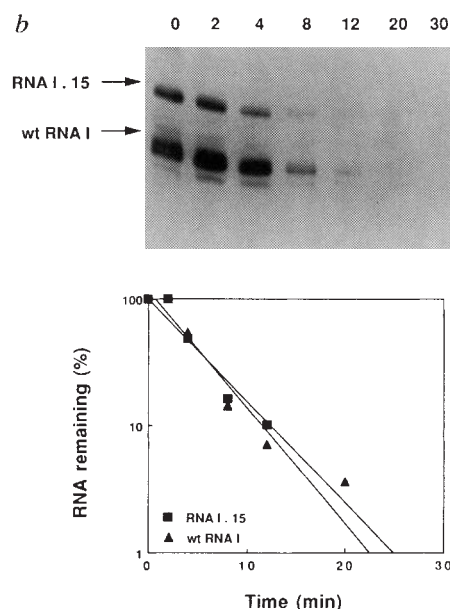
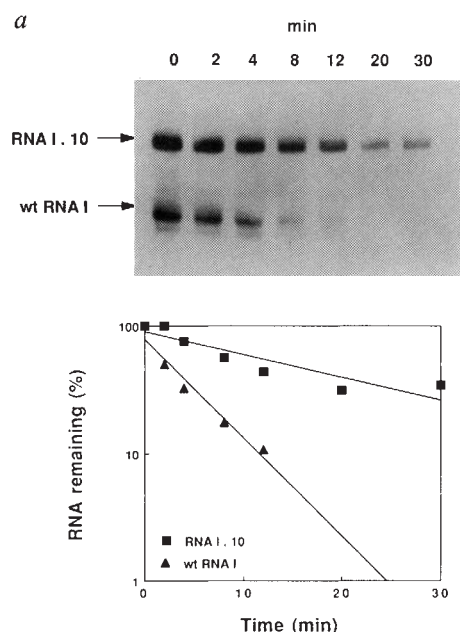


FIG. 2 Degradation of RNA I.10, RNA I.15 and RNA I.15ΔE. At time intervals after transcription inhibition with rifampicin ($200 \mu\text{g ml}^{-1}$), total cellular RNA was isolated from *E. coli* strain N3433⁷ containing pPB10 (a), pPB15 (b) or pPB15ΔE (c) and growing exponentially in M9-glucose medium at 35 °C. The RNA samples were subjected to northern blot analysis²⁵ with a single-stranded RNA probe complementary to RNA I. Bands that correspond to each RNA I variant and to wild-type (wt) RNA I are indicated. Beneath each autoradiogram is a semilogarithmic plot of RNA concentration as a function of time. See Table 1 for measured half-lives.

METHODS. All plasmids used for half-life measurements were derivatives of pBR322 and encoded both wild-type RNA I and a mutant RNA I transcribed from the *bla* promoter. A *Bst*UI fragment of pBR322 (base pairs, 2,521–3,102) encoding RNA I was inserted into the *Sma*I site of M13mp18 so as to delete the RNA II promoter and acquire a *Pst*I site downstream of the RNA I transcription terminator. By oligonucleotide-directed mutagenesis, a *Bcl*I site was created at the RNA I transcription initiation site: GAAGGA → TGATCA (the first transcribed nucleotide of wild-type RNA I is underlined). The resulting 0.16 kilobase (kb) *Bcl*I–*Pst*I fragment encoding RNA I was inserted between the corresponding sites of pBLA200¹⁷ to create pPB4, which encodes RNA I.4 transcribed from the *bla* promoter. Insertion of the same *Bcl*I–*Pst*I fragment between the *Bam*HI and *Pst*I sites of pT7/T3α19 generated pT7/T3RNAI. Other plasmids used for half-life measurements were derived from pPB4; numerical designations in the names of these plasmids correspond to the RNA I allele that each plasmid encodes. RNA isolation, Northern blotting, and quantitative analysis of data were done as described^{17,25}. Northern blots were probed with radiolabelled RNA synthesized *in vitro* with T7 RNA polymerase, using *Eco*RI-linearized pT7/T3RNAI as the template.

mutation in the *rne* gene (Table 1), which encodes a protein necessary for RNase E activity *in vivo* and *in vitro*⁷⁻¹⁶. Furthermore, deletion of seven nucleotides (AGUAUUU) surrounding the RNase E cleavage site of RNA I.15 increases the RNA half-life from 3.0 min to 13 min (RNA I.15ΔE, Fig. 2c). Therefore, the five unpaired nucleotides at the 5' end of RNA I.15 and RNA I.25 must destabilize the RNA by increasing the vulnerability of the normal RNase E cleavage site, rather than by creating a new cleavage site for RNase E or another ribonuclease.

Another experiment showed directly that a 5'-terminal stem-loop impedes cleavage at the internal RNase E cleavage site. In theory, modifications that slow initial cleavage of RNA I should reduce the steady-state concentration of the cleavage products relative to that of their full-length precursor. The relative level of the 3' RNase E cleavage product versus the full-length transcript was determined by primer extension analysis for RNA I.20, RNA I.25, and wild-type RNA I (Fig. 3b). As expected, this concentration ratio was much lower for RNA I.20 than for RNA I.25 and wild-type RNA I. In accord with kinetic theory, although full-length RNA I.20 is much more abundant than RNA I.25 because of its greater stability, the steady-state concentrations of their respective 3' cleavage products are very similar because of the proportionately higher rate constant for cleavage of RNA I.25 by RNase E.

Together, these data indicate that RNase E preferentially

degrades RNAs that begin with several unpaired nucleotides at the 5' end. As cleavage by RNase E is thought to be the rate-determining step in the decay of many *E. coli* messages⁹⁻¹⁶, this preference could explain how rates of prokaryotic mRNA degradation can be controlled by elements near the 5' end¹⁻⁵ despite the apparent absence of bacterial 5' exoribonucleases⁶. Furthermore, as RNA I is not translated in *E. coli*, our findings show that RNA stabilization by a 5'-terminal stem-loop is not mediated by the quantity or quality of translating ribosomes, but must instead be a direct consequence of the properties of RNase E.

For RNAs that begin with a single-stranded segment upstream of the RNase E cleavage site, the rate of degradation by RNase E seems to be independent of the sequence of this segment, the distance from the 5' terminus to the internal cleavage site (for distances up to 42 nt), and the ability of the intervening nucleotides to form secondary structures. Just five unpaired bases at the 5' end are sufficient to allow rapid degradation of RNA I and *ompA* mRNA, whereas two unpaired bases at the 5' end of *ompA* mRNA are not destabilizing¹⁹.

We propose that RNase E may act by first binding preferentially to a single-stranded RNA 5' end or to another protein already bound there, and then cleaving at suitable sites downstream. The accessibility of RNase E cleavage sites, as determined by base pairing at the 5' terminus, apparently can be as important as their intrinsic vulnerability to cleavage in control-

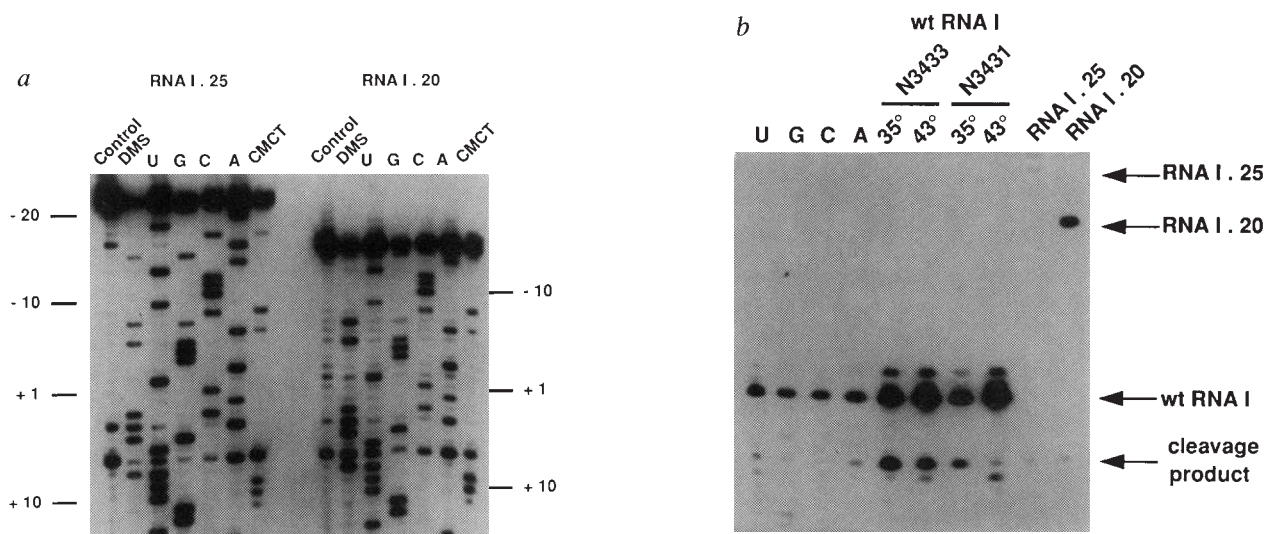


FIG. 3 Inhibition of RNase E cleavage by a 5'-terminal stem-loop. *a*, Comparative structural analysis of RNA I.20 and RNA I.25. These two RNA I variants were synthesized *in vitro* and alkylated with either DMS or CMCT^{18,19}. Sites of alkylation were mapped by primer extension^{18,19}, using a complementary oligodeoxynucleotide (5'-TCAAGAGCTACCAACTC-3'). Gel electrophoresis was done in parallel with the products of primer extension on unalkylated templates in the presence (lanes U, G, C, A) or absence (control lane) of dideoxynucleoside triphosphates. The sequencing lanes are labelled to indicate the sequence of the RNA, not the complementary DNA. Calibration is in nucleotides from the 5' end of wild-type RNA I. Blockage of primer extension by an alkylated RNA base results in a complementary DNA fragment that is 1 nt shorter than that arising from incorporation of a dideoxynucleotide opposite the same RNA base. Alkylatable bases (upper-case letters) within the 5'-terminal 39-nt segment of RNA I.25 are as follows: gAucAgauccUcGAgggauCAGuAUUggu; although not detectably alkylated *in vitro*, the first adenosine residue in RNA I.25 is methylated by DMS *in vivo* (data not shown). The degree of alkylation of the uridine at position +5 could not be determined because of some premature termination there by reverse transcriptase on an unalkylated template. The alkylatable bases in RNA I.20 are the same as those in RNA I.25 except that the first five nucleotides are absent. *b*, Relative efficiency of RNase E cleavage of RNA I, RNA I.20, and RNA I.25 in *E. coli*. Total cellular RNA was isolated from *E. coli* strain N3433 containing pPB1020 or pPB1025 and growing exponentially in M9-glucose medium at 35 °C. These two plasmids encode RNA I.20 and

RNA I.25, respectively, but not wild-type RNA I. RNA was also isolated from strains N3431 (*rne*^{ts}) and N3433 (*rne*⁺) containing pBR322 before and after a temperature upshift from 35 °C to 43 °C. In each case, the steady-state concentration of the RNase E cleavage product of RNA I, RNA I.20, or RNA I.25 was compared to that of the corresponding intact precursor transcript by primer extension analysis of 3 µg of cellular RNA. Primer extension in the presence of dideoxynucleotides generated a set of sequence ladders (lanes U, G, C, A). Because of promoter and plasmid copy number differences, the cellular concentration of wild-type RNA I from pBR322 is higher than the concentration of RNA I.20 and RNA I.25 from pPB1020 and pPB1025. METHODS. Plasmids for *in vitro* synthesis of RNA I.20 (pT7-R20) and RNA I.25 (pT7-R25) were obtained by subcloning portions of pPB20 and pPB25 into pSE18¹⁸. Fnu4HI fragments of pT7-R20 and pT7-R25 were then used as templates for *in vitro* synthesis of RNA I.20 and RNA I.25 by T7 RNA polymerase. The resulting RNAs each had one more nucleotide (G) at the 5' end and eight more nucleotides (UGCAAGCA) at the 3' end than the corresponding RNAs synthesized *in vivo*. Before alkylation, these RNAs were purified by gel electrophoresis. To construct plasmids pPB1020 and pPB1025, a 0.8 kb *Hae*III fragment containing the replication origin of plasmid pMF35³² was ligated to a 1.7 kb *Xmn*I-*Hinc*II fragment containing the *cat* gene of pACYC184, and a 1.3 kb *Sal*I fragment of pPB20 or pPB25 was then inserted into the *Sal*I site of the resulting plasmid (pPB1000). Replication of pPB1020 and pPB1025 in N3433 requires the presence of a helper plasmid (Δ22-*pir*116)³².

TABLE 1 Half-lives of mutant RNA I transcripts

RNA I allele	RNA structure†	Mutant RNA I half-life (min)‡	Wild-type RNA I half-life (min)‡
RNA I (wild-type)			2.7 ± 0.4
RNA I.10		13 ± 2	2.8 ± 0.3
RNA I.20		13 ± 2	3.1 ± 0.3
RNA I.30		7.0 ± 1.0	3.1 ± 0.4
RNA I.40		13 ± 2	2.9 ± 0.2
RNA I.4		2.6 ± 0.2	3.2 ± 0.5
RNA I.15		3.0 ± 0.5	2.6 ± 0.2
RNA I.25		2.1 ± 0.2	2.3 ± 0.2
RNA I.15ΔE		13 ± 1	2.6 ± 0.5

† The expected secondary structure of each RNA I mutant is represented diagrammatically (for a detailed secondary structure of wild-type RNA I, see Fig. 1). I, II, III, RNA I stem-loops. *, Synthetic hairpin hp*. X, synthetic hairpin hpX. 1, *ompA* hairpin hp1 (GAUCACACAGGGGUGCUCGCAUAAGCGAAGAU-AUCGGUAGAGUUAUAUUGAGCAGAUCCCCGUGAUC)¹⁹. Arrow, RNase E cleavage site^{24,25}. RNA I.10 is RNA I with hp* added at the 5' terminus. RNA I.20 is RNA I with hpX added at the 5' terminus. RNA I.30 is RNA I with *ompA* hp1 added at the 5' terminus. RNA I.40 is RNA I.10 with four unpaired nucleotides (GAUC) added immediately downstream of hp*. RNA I.4 is RNA I with four unpaired nucleotides (GAUC) added at the 5' terminus. RNA I.15 is RNA I.10 with five unpaired nucleotides (GAUCC) added upstream of hp*. RNA I.25 is RNA I.20 with five unpaired nucleotides (GAUCA) added upstream of hpX. RNA I.15ΔE is RNA I.15 with a deletion of seven internal nucleotides (AGUAAUU) surrounding the RNase E cleavage site.

‡ The half-life of each mutant RNA I and of wild-type RNA I was measured simultaneously at 35 °C in *E. coli* strain N3433 (*rne*⁺, *lacZ43*, *relA*, *spoT1*, *thi1*)⁷ containing a plasmid that encodes both RNAs. See Fig. 2 for experimental details. The half-life of RNA I.15 increases to 22 ± 2 min in the isogenic *rne*^{ts} *E. coli* strain N3431 (*rne*-3071^{ts}, *lacZ43*, *relA*, *spoT1*, *thi1*)⁷ within 30 min after a temperature upshift from 35 to 43 °C (half-life of wild-type RNA I = 14 ± 1 min; its half-life at 43 °C in N3433 is 3.3 ± 0.2 min (half-life of wild-type RNA I = 3.7 ± 0.2 min). The half-life of RNA I.25 increases to 18 ± 1 min in N3431 after a temperature upshift to 43 °C (half-life of wild-type RNA I = 11 ± 1 min; its half-life at 43 °C in N3433 is 5.6 ± 0.4 min (half-life of wild-type RNA I = 5.6 ± 0.9 min).

ling the rate of RNA degradation by RNase E *in vivo*. Although RNase E cleavage can be inhibited by a 5'-terminal stem-loop, it is not abolished completely, as RNA I.10 and RNA I.20 are slowly cleaved *in vivo*. This finding suggests either that RNase E can bypass the 5' end with low efficiency or that RNase E can interact, albeit less effectively, with a base-paired 5' terminus. The latter interpretation might explain why not all 5' stem-loops are equally effective barriers to degradation by this enzyme.

The ability of 5'-terminal unpaired nucleotides to aid RNase E cleavage at a downstream cleavage site in an untranslated RNA suggests that this enzyme has an intrinsic orientation of digestion that might explain the many previous reports of 5'-to-3'

mRNA decay in prokaryotes²⁶⁻³¹. Such an explanation obviates any need to invoke ribosome movement or the existence of an undiscovered bacterial 5' exoribonuclease to account for this phenomenon. □

Received 4 May; accepted 9 October 1992.

1. Yamamoto, T. & Imamoto, F. *J. molec. Biol.* **92**, 289-309 (1975).
2. Gorski, K., Roch, J.-M., Prentki, P. & Krusch, H. M. *Cell* **43**, 461-469 (1985).
3. Belasco, J. G., Nilsson, G., von Gabain, A. & Cohen, S. N. *Cell* **46**, 245-251 (1986).
4. Bechhofer, D. H. & Dubnau, D. *Proc. natn. Acad. Sci. U.S.A.* **84**, 498-502 (1987).
5. Sandler, P. & Weisblum, B. *J. molec. Biol.* **203**, 905-915 (1988).
6. Deutscher, M. P. & Zhang, J. in *Post-transcriptional Control of Gene Expression* (eds McCarthy, J. E. G. & Tuite, M. F.) 1-11 (Springer, Berlin, 1990).
7. Goldblum, K. & Apirion, D. *J. Bact.* **146**, 128-132 (1981).
8. Roy, M. K. & Apirion, D. *Biochim. biophys. Acta* **747**, 200-208 (1983).
9. Apirion, D. *Genetics* **90**, 659-671 (1978).
10. Ono, M. & Kuwano, M. *J. molec. Biol.* **129**, 343-357 (1979).
11. Lundberg, U., von Gabain, A. & Meleforts, O. *EMBO J.* **9**, 2731-2741 (1990).
12. Mudd, E. A., Krusch, H. M. & Higgins, C. F. *Molec. Microbiol.* **4**, 2127-2135 (1990).
13. Babitzke, P. & Kushner, S. R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1-5 (1991).
14. Meleforts, O. & von Gabain, A. *Molec. Microbiol.* **5**, 857-864 (1991).
15. Taraseviciene, L., Miczak, A. & Apirion, D. *Molec. Microbiol.* **5**, 851-855 (1991).
16. Mackie, G. J. *Bact.* **173**, 2488-2497 (1991).
17. Emory, S. A. & Belasco, J. *J. Bact.* **172**, 4472-4481 (1990).
18. Chen, L.-H., Emory, S. A., Bricker, A. L., Bouvet, P. & Belasco, J. G. *J. Bact.* **173**, 4578-4586 (1991).
19. Emory, S. A., Bouvet, P. & Belasco, J. G. *Genes Dev.* **6**, 135-148 (1992).
20. Tomizawa, J.-I., Itoh, T., Selzer, G. & Som, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1421-1425 (1981).
21. Polisky, B. *Cell* **55**, 929-932 (1988).
22. Morita, M. & Oka, A. *Eur. J. Biochem.* **97**, 435-443 (1979).
23. Tamm, J. & Polisky, B. *Nucleic Acids Res.* **11**, 6381-6397 (1983).
24. Tomcsanyi, T. & Apirion, D. *J. molec. Biol.* **185**, 713-720 (1985).
25. Lin-Chao, S. & Cohen, S. N. *Cell* **65**, 1233-1242 (1991).
26. Morikawa, N. & Imamoto, F. *Nature* **223**, 37-40 (1969).
27. Morse, D. E., Mosteller, R. D., Baker, F. & Yanofsky, C. *Nature* **223**, 40-43 (1969).
28. Forchhammer, J., Jackson, E. N. & Yanofsky, C. *J. molec. Biol.* **71**, 687-699 (1972).
29. Cannistraro, V. J. & Kennell, D. J. *Bact.* **161**, 820-822 (1985).
30. Bechhofer, D. H. & Zen, K. H. *J. Bact.* **171**, 5803-5811 (1989).
31. Sandler, P. & Weisblum, B. *J. Bact.* **171**, 6680-6688 (1989).
32. Wu, F., Goldberg, I. & Filutowicz, M. *Nucleic Acids Res.* **20**, 811-817 (1992).

ACKNOWLEDGEMENTS. We thank S. Cohen and S. Lin-Chao for discussions and M. Filutowicz for providing plasmids pMF35 and Δ22-*pir116*. This research was supported by a grant from the NIH to J.G.B. and by funds from the Association pour la Recherche sur le Cancer to P.B.

ERRATA

Abrogation by c-myc of G1 phase arrest induced by RB protein but not by p53

David W. Goodrich & Wen-Hwa Lee

Nature **360**, 177-179 (1992)

IN this letter in the 12 November issue, the two-part Fig. 1 and single-part Fig. 2 were transposed. The figure legends are correct as printed.

Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages

Peter Mombaerts, Alan R. Clarke, Michael A. Rudnicki, John Iacomini, Shigeyoshi Itohara, Juan J. Lafaille, Lili Wang, Yoshiaki Ichikawa, Rudolf Jaenisch, Martin L. Hooper & Susumu Tonegawa

Nature **360**, 225-231 (1992)

IN Fig. 3 of this article the layout of parts a and b on page 227 was misleading. The three panels in part b correspond to the first three panels in part a, that is, WT, α-/- and β-/- respectively. In addition, the last sentence in paragraph 4 of the Discussion on page 231 should read: 'However, rearrangements may occur sequentially at the TCR-α loci, but without a causal relationship.'

Imprinting and splicing join together

A gene thought to be involved in RNA splicing, and which is imprinted in mice, may play an important part in Prader-Willi syndrome

THE phenomenon of genetic imprinting holds a particular fascination for biologists. By what mechanism, and for what purpose, should some genes (which ones?) be expressed only from the chromosome inherited from the mother, and others only from the paternal chromosome? The best known examples in humans are the widely contrasting phenotypes of the Prader-Willi and Angelman syndromes, both of which are typically caused by deletions of the same portion of the long arm of chromosome 15 (15q11-q13). Patients with Prader-Willi syndrome (PWS) (who have either a paternal deletion or two copies of the mother's chromosome 15)¹ suffer from obesity and assorted developmental defects, whereas children with Angelman syndrome (AS) (with a maternal deletion or paternal uniparental disomy)² are mentally retarded with puppet-like movements, and are prone to seizures.

It is highly likely that a number of imprinted genes contribute to the two syndromes, with PWS, for example, resulting from the paternal loss of a maternally imprinted gene(s). In an effort to define those genes, work has progressed on two main fronts — characterizing rare affected families with unusual deletions which may sublocate the critical regions responsible for the two disorders^{3,4}, and transposing this knowledge into a physical map of the region⁵. The upshot of these studies has been that the genes involved in PWS seem to lie closer to the centromere of chromosome 15 than those that make up the AS region, being spread over one megabase (Mb) or more of DNA, whereas the AS critical region is somewhat shorter. But so far the only known gene that maps to either interval is that for the γ -aminobutyric acid receptor B3, which lies in the AS interval.

In two papers in this month's *Nature*

Also in this month's *Nature Genetics*: three reports on the linkage of early-onset familial Alzheimer's disease to chromosome 14; the role of mitochondrial DNA deletions in the brain in ageing; repetitive sequences and a PMP-22 mutation in Charcot-Marie-Tooth disease; chromosome fragmentation using telomeres for genome analysis; and the effects of trinucleotide-repeat expansion in Kennedy's disease.

Genetics, the groups of Uta Francke and Stuart Leff at Stanford, and Neal Copeland and Nancy Jenkins at the National Cancer Institute, show that a newly discovered imprinted gene in mice, whose product is implicated in messenger RNA processing, maps to the critical genomic region involved in PWS^{7,8}. And in an accompanying paper, Bruce Cattanach and colleagues show that this imprinted gene is not expressed in a mouse model for PWS that they have constructed⁹.

The gene studied by these workers, *SNRPN*, encodes the small nuclear ribonucleoprotein polypeptide N (SmN). SmN is a component of the small nuclear ribonucleoprotein particles (SnRNPs) found in spliceosomes, which catalyse the splicing and processing of mRNA. SnRNPs are composed of one of five small nuclear RNAs (U1, U2, U4, U5 and U6) and a number of associated polypeptides. Some, such as SnRNP B, are ubiquitously expressed, but others, including the closely related SmN, are tissue-specific. SmN is found predominantly in the brain and central neurons, and as such proteins are known to interact with mRNA, it is tempting to predict a role for SmN in the tissue-specific control of RNA splicing. Özcelick *et al.*⁸ cloned the SmN gene and mapped it precisely on chromosome 15 by determining the presence or absence of *SNRPN* in DNA from patients with deletions of the PWS/AS region. Every PWS patient with a chromosomal deletion was also missing one copy of *SNRPN*, including two PWS patients with partial, overlapping deletions.

These results firmly place *SNRPN* in the PWS critical region on chromosome 15q12 — the first known gene to be so assigned. The significance of this result is strengthened by the accompanying work, which shows that the murine homologue, *Snrpn*, is maternally imprinted (expressed exclusively from the paternal allele)⁷. Leff *et al.* mapped the *Snrpn* gene to the central portion of mouse chromosome 7, which is syntenically conserved with human chromosome 15q11-13. Next, using an imprinting assay¹⁰ that distinguishes between the transcripts from two alleles in a cross between *Mus musculus* and *Mus spretus*, they could show that in brain RNA only the male-derived *Snrpn* transcript is detected. The maternal imprinting of *Snrpn* in mice does not necessarily mean that the human *SNRPN*

gene is imprinted as well, but as at least one known imprinted murine gene, *H19*, has been shown to be monoallelically expressed in humans¹¹, it is a legitimate proposal.

The putative role for *SNRPN* in PWS is further supported by work from Cattanach and colleagues⁹, who have created a mouse model for the disease in which mice possess two maternally derived copies of the central portion of chromosome 7. These mice fail to thrive and suffer early postnatal lethality, which may correspond to the early feeding difficulties of PWS infants. No *Snrpn* message could be detected in the brains of mice with the maternal duplication shortly after birth, whereas normally the transcript is very abundant. In contrast, mice with a paternal duplication of a larger region from chromosome 7 were much healthier and without serious abnormality, although their thin, frail bones could be taken as a sign of an AS-like phenotype.

Could a lack of *SNRPN* expression contribute to PWS? One possibility is that SmN normally regulates the splicing of a number of neural-specific genes which might then be affected in PWS. If a role for SmN is borne out, it would be one of the first examples in which a gene product involved in the DNA → RNA → protein pathway (the 'central dogma' of molecular biology) has been found. Such a possibility has been aired by David Page and colleagues, who have suggested that the ribosomal S4 proteins encoded by genes on the X and Y chromosomes may be involved in the Turner's syndrome phenotype¹².

Kevin Davies

Kevin Davies is Editor of Nature Genetics.

- Nicholls, R. D. *et al.* *Nature* **342**, 281-285 (1989).
- Malcolm, S. *et al.* *Lancet* **337**, 694-697 (1991).
- Hamabe, J. *et al.* *Am. J. med. Genet.* **41**, 64-68 (1991).
- Wagstaff, J. *et al.* *Nature Genet.* **1**, 291-294 (1992).
- Kuwano, A. *et al.* *Hum. molec. Genet.* **1**, 417-425 (1992).
- Wagstaff, J. *et al.* *Am. J. hum. Genet.* **49**, 330-337 (1990).
- Leff, S. E. *et al.* *Nature Genet.* **2**, 265-269 (1992).
- Özcelick, T. *et al.* *Nature Genet.* **2**, 265-269 (1992).
- Cattanach, B. M. *et al.* *Nature Genet.* **2**, 270-274 (1992).
- Zhang, Y. & Tycko, B. *Nature Genet.* **1**, 40-44 (1992).
- Bartolomei, M. S., Zemel, S. & Tilghman, S. M. *Nature* **351**, 153-155 (1991).
- Fisher, E. M. C. *et al.* *Cell*, **63**, 1205-1218 (1991).

Making light work with optical tweezers

Steven M. Block

Microscopic objects, including biological material, can be remotely manipulated with tightly focused beams of infrared laser light. The use of optical traps, or 'optical tweezers', holds great promise for noninvasive micromanipulation and mechanical measurement in cell biology. Optical tweezers are the 'tractor beams' of today's technology.

LIGHT is fundamental to the study of biophysical phenomena, primarily because it is so versatile and can be controlled with such extraordinary spatial and temporal precision. Light forms the basis of microscopy and spectroscopy. When used in conjunction with a burgeoning arsenal of fluorescent and phosphorescent probes, it can measure a range of cellular properties, including voltage, ion concentrations, spatial distributions of molecules, etcetera. Light-sensitive compounds can perform useful photochemistry, activating, inactivating, crosslinking, and synthesizing biomolecules. Sensitive light-based sensors are rapidly replacing radioisotopes for many applications. The availability of intense, spectrally pure laser light sources, coupled with new molecular approaches, has fostered a revolution in optical biotechnology. Confocal scanning and atomic force microscopes, gene sequencers, cell sorters and a host of recent technologies all rely upon laser light. In the midst of this revolution comes another laser-based tool that takes advantage of an unlikely property of light, namely its ability to produce radiation pressure. Absorption, reflection or refraction of light by dielectric material (which could be biological in origin) generates tiny forces. Such forces are minuscule indeed: several milliwatts of power, corresponding to a bright laser beam, give only a few piconewtons ($1 \text{ pN} = 10^{-12} \text{ N} = 0.1 \text{ } \mu\text{dyn}$). However, a force of 10 pN is sufficient to pull a bacterium such as *Escherichia coli* through water ten times faster than it can swim. So in the microscopic realm, radiation pressure could play a significant role — at least in principle.

Putting light to work

Now for the practice. A few years back, Ashkin and colleagues at AT & T Bell Laboratories proposed a laser-based optical trap for microscopic particles¹. Ashkin went on to demonstrate that it could manipulate living material noninvasively, including viruses, bacteria, yeast and protozoa^{2,3}. This invention makes use of the so-called 'gradient force' that appears when a transparent material with a refractive index greater than the surrounding medium is placed in a light gradient. As light passes through polarizable material, it induces fluctuating dipoles. These dipoles interact with the electromag-

netic field gradient, resulting in a force directed towards the brighter region of the light. (Conversely, an object with a refractive index lower than the surrounding medium, such as an air bubble in water, experiences a force drawing it towards the darker region.) A ray-optic representation of the gradient force, valid when particles are larger than the wavelength of light, is shown in Fig. 1. Parallel rays of light enter a small, refractile sphere from above and are bent because it acts as a lens. Before entering, rays travel vertically and carry no horizontal momentum. After deflection, however, they pick up horizontal momentum. By conservation of momentum in the horizontal direction, an equal and opposite momentum change is conveyed to the sphere, much as a turbine blade experiences a reaction force from the fluid it deflects. If the intensity profile in the incoming beam were uniform, the reaction forces on the left and right sides of the sphere would cancel and there would be no net sideways component. In a gradient, however, the asymmetry in the light gives rise to an imbalance in the reaction forces and the object is pulled towards the bright side. (Along with the gradient force, of course, there is the 'traditional' scattering force, which arises when light is scattered by an object: this force component tends to push objects along the direction of light propagation, which is not necessarily in the direction of the light gradient.)

Optical tweezers

To make an optical trap based on the gradient force, one simply constructs a three-dimensional light gradient with the brightest light in the centre. Such gradients occur near any focus: the sharper the focus, the steeper the gradient. In practice, the steepest possible gradients are needed to ensure that the gradient force overcomes the scattering force near the focal zone, so that the object will not be ejected from the trap along the direction of the beam. Sufficiently steep gradients can be achieved by focusing laser

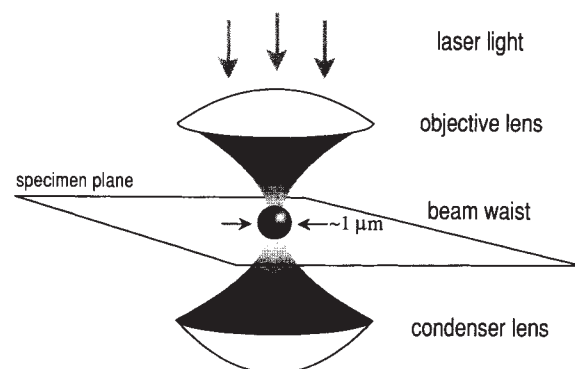


FIG. 1 Schematic of optical tweezers. Laser light enters the objective lens of a microscope from above and is focused to a diffraction-limited beam waist in the specimen plane, creating a three-dimensional light gradient: a micrometre-sized object is trapped. Microscope illuminating rays that fill the specimen plane (not shown) generally come from below.

light to a diffraction-limited spot of diameter $\sim \lambda$, the light wavelength, through a microscope objective of high numerical aperture (Fig. 2). This scheme constitutes the single-beam gradient force optical trap, aptly dubbed 'optical tweezers'. Near a diffraction-limited spot, however, the ray-optic picture breaks down and accurate calculations of the trapping forces on small particles become quite complex: these have not been attempted except for the simplest geometries⁴⁻¹¹. Other distributions of incident light can also create two- or three-dimensional traps, and the scattering of light by illuminated objects can produce mutual forces among the objects themselves, dubbed optical binding forces, giving rise to what has been termed 'optical matter'¹². Optical scattering, gradient and binding forces are all manifestations of the same underlying physics.

The characteristic grasp of optical tweezers is roughly given by λ , but objects ranging from tens of nanometres to many micrometres in size can be manipulated by optical tweezers. For stable trapping, laser powers from several milliwatts up to several watts are used, producing tens to hundreds of piconewtons on micrometre-sized objects: the force is proportional to the light power. At these enormous fluxes, light absorption by the trapped object can result in excessive heating or photodamage. To minimize such effects, Ashkin and colleagues chose a convenient wavelength in the near

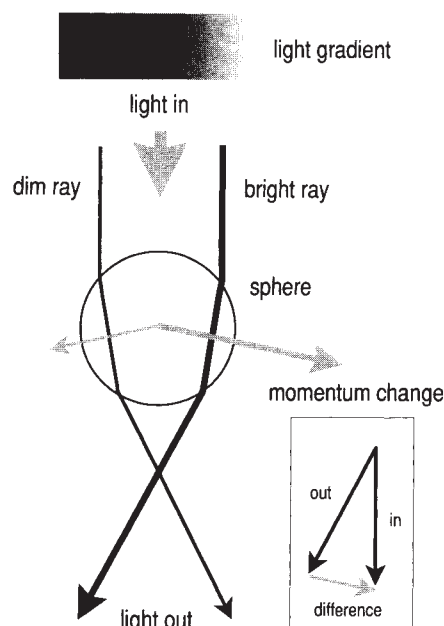


FIG. 2 Origin of the gradient force. A parallel beam of light with a gradient in intensity (depicted by the shaded region) impinges on a spherical lens. Two representative rays are drawn (black arrows), their relative thickness symbolizing intensity. The rays are bent, giving rise to the reaction forces shown acting through the centre of the sphere (grey arrows). The resultant of all rays pulls the sphere towards the brighter light. Inset: a vector diagram showing the momentum change in the bright ray.

infrared (1,064 nm, produced by a Nd:YAG laser) that is poorly absorbed by biological tissue, and most subsequent work has been done at this wavelength. The advent of convenient, low-cost diode lasers in the near infrared region (780–950 nm) makes these devices particularly attractive for use at the low power end¹³ and the first commercial version of optical tweezers based on such a solid-state device has recently been brought to market (LaserTweezers, Cell Robotics, Inc., Albuquerque, New Mexico). Other near-infrared lasers, especially the titanium–sapphire laser, may afford the right combination of power and wavelength for biological use at higher power. (For reviews of optical tweezers, see refs 14–18.)

Micromanipulators/tensiometers

As micromanipulators, optical tweezers have several advantages over conventional devices. They can be combined with any number of microscope imaging modes, including brightfield, phase contrast, differential interference contrast and epifluorescence. They are relatively free of creep, backlash and hysteresis, and they remain parfocal with the specimen. They can be steered with great rapidity and their grasp can be released instantly. They can be made to pull with variable force. The forces delivered to isolated objects are reproducible and readily calibrated, and optical traps can be easily adapted to the measurement of

extremely small tensions (see below). Optical tweezers work within sealed preparations and with double-oil immersion optics, permitting the highest possible resolution, down to the diffraction limit. They can reach right through cell or organelle walls to grasp objects inside. Living organisms can be maintained for periods of seconds to hours, depending upon their tolerance for high levels of infrared light. On the downside, extreme light levels lead to 'optication' of the preparation (a colourful term coined by Ashkin) and, in any case, the absolute forces remain quite small and confined to a micrometre-sized region. Such limitations will prevent optical tweezers from ever replacing, for example, the suction pipettes used to hold cells for microinjection.

A potpourri of uses

Piconewtons, however, are well matched to the kinds of forces encountered at the level of biological ultrastructure. Forces of this magnitude can move cells, bend cytoskeletal elements, such as microtubules, distort proteolipid membranes, arrest organelle movements or swimming bacteria and overcome the motion of biological motors, such as myosin, kinesin and the bacterial rotary engine. They are probably sufficient to disrupt some macromolecular bonds, such as receptor–ligand complexes. Several recent studies have shown the utility of optical tweezers. Soon after demonstrating that cells could be manipulated using infrared light, Ashkin and Dziedzic used optical tweezers to grasp membranes inside plant cells and pull out slender viscoelastic filaments¹⁹. These strands could be stretched, moved about and occasionally fused with other membranes, altering the structure of the cell and enabling a kind of cellular microsurgery. By carefully calibrating the optical force against Stokes' drag at varying light levels, Block, Blair and Berg made a micromechanical measurement of the torsional compliance of single bacterial flagella in *E. coli* and *Streptococcus*²⁰. This compliance was shown to be associated with the hook, a flexible coupler consisting of only ~150 protein monomers polymerized into a short tube that connects the 'drive shaft' of the bacterial motor to the flagellar filament²¹. Optical tweezers later helped to show that periplasmic flagella of spirochaete bacteria, which are normally confined internally between the cell wall and inner membrane, are also driven by rotary motors²², as had been proposed long ago by Berg²³.

Optical tweezers can be combined with other laser-based techniques, notably pulsed ultraviolet microbeams ('optical scalpels'), which cut or ablate biological material on a microscopic scale. Weigand-Steubing, working in the laboratories of Greulich and Berns, accomplished laser-assisted cell fusion by bringing together two cells with optical tweezers and perforating their apposed membranes with UV light²⁴. Research-

ers in Greulich's laboratory have pursued further applications, including the use of optical tweezers in genome mapping, to collect and isolate specific chromosome fragments that have been microdissected by UV microbeams, and in immunology, by placing natural killer cells against target erythroleukaemia cells²⁵. Berns and colleagues have used optical tweezers, sometimes combined with optical scalpels, to study the motion of chromosomes during mitosis^{26,27}, as well the swimming force of human sperm²⁸. Their laboratory has also tried using optical tweezers for *in vitro* fertilization. Recently, they began an investigation of photodamage over a range of wavelengths using a tunable titanium–sapphire laser and showed that, for a given power, it produced substantially less photodamage at 700 nm than the Nd:YAG laser at 1,064 nm: this bodes well for future studies requiring larger forces or smaller objects²⁹.

Optical traps hold particular promise for the study of motor molecules, where the forces generated by these mechanoenzymes are believed to be in the range of a piconewton or so. One especially powerful approach has been to use micrometre-sized spheres as 'handles' to hold molecules or molecular assemblies. Refractile silica or latex spheres can be strongly trapped and their symmetry and uniformity facilitate calibration against Stokes' drag. Shepherd *et al.* used optical tweezers for an *in vitro* motility assay, capturing freely-diffusing, myosin-coated latex beads out of solution and placing them against actin cables isolated from hair-cell stereocilia³⁰. These beads bound and moved unidirectionally along the actin in the presence of ATP and could be tracked with high precision. Similarly, Block *et al.* showed that kinesin-coated silica beads could be placed directly on axonemes or microtubules, greatly improving the efficiency of *in vitro* bead assays, and demonstrated movement by single kinesin molecules³¹. Ashkin and coworkers used optical tweezers to arrest and then release small organelles powered by attached motors in the giant amoeba *Reticulomyxa*, thereby obtaining an estimate of motor force³². Kuo and Sheetz have undertaken a related approach with latex spheres to estimate the force produced by kinesin along microtubules³³.

Small spherical 'handles' serve other purposes as well. Edidin *et al.* and Kucik *et al.* used latex or gold particles attached to transmembrane proteins to probe the mobility of these complexes in the plane of the membrane³⁴ and their transport by the cytoskeleton³⁵. By attaching microspheres to the ends of DNA made fluorescent with ethidium bromide, Chu and colleagues stretched out single molecules and released them: this relaxation was used to estimate the persistence length of the polymer³⁶. Svoboda *et al.* attached latex particles covalently to ghosts of human red blood

cells and then used optical tweezers to hold these ghosts away from surfaces while detergent was washed past inside a microscope flow chamber³⁷. This treatment dissolves away the lipid part of the membrane, leaving behind the labile spectrin skeleton, which could then be studied in the absence of complicating surface interactions. Spherical handles attached to cell surfaces provide enough purchase to grasp membranes and pull them away from the cell, forming thin structures that are reminiscent of filopodia, or of the viscoelastic filaments reported by Ashkin. Sheetz's group calls these structures 'neopodia' and is using them to study the reorganization of cytoskeletal elements³⁸.

Recent developments have expanded the utility of optical tweezers. Optical trapping beams can be steered with lenses or galvanometer mirrors, much as is done in the scanning confocal microscope¹⁵. Brakenhoff and Visscher have taken this one step further, generating multiple traps by illuminating a single region with the laser for a moment, then rapidly moving the beam elsewhere in the specimen and illuminating a second region, then moving on to the next location, and so on. As many as eight separate traps can be made with one laser beam by this 'time-sharing' approach (G. J. Brakenhoff, personal communication). Multiple traps permit objects of different shapes and sizes to be spatially oriented, pulled, and so forth, in ways that are not possible with single traps. Simpler double-trap arrangements have recently been used to study stochastic resonance phenomena associated with thermal hopping over time-varying potential barriers³⁹ and to assemble arrays of latex particles into ordered structures⁴⁰.

New wrinkles

A three-way collaboration among the groups of Spudich and Chu at Stanford University, California and Simmons at King's College, London has developed a second-generation optical tensiometer based on traps. In their scheme, the position of a bead in an optical trap is monitored at high precision by a split photodiode detector, the output of which is

used in a feedback loop to servo the position of the trap using an acousto-optic modulator to deflect the beam. This arrangement effectively stiffens the trap, turning it into an approximately isometric tensioning device. The level of feedback signal required to close the loop in this condition provides a measure of the force generated by the trapped object. Spudich, Chu, Simmons and coworkers hope to measure the force produced by myosin motors as they move against actin filaments, among other things⁴¹. Their plans call for two traps to be servoed, so that a floppy actin filament can be levitated and stretched taut between two bead handles. Yanagida and coworkers at Osaka University have also built a two-beam arrangement for holding an actin filament suspended, a geometry that offers many experimental possibilities. My group has constructed an instrument combining the dual-beam interferometer invented by Denk and Webb⁴² with optical tweezers, resulting in a hybrid device that can measure the displacement of a trapped object down to distances as small as Ångströms, thousands of times per second, using polarized light. Novel trap designs should provide new ways of getting down to the molecular level with biological systems. Bright times shine ahead for optical traps. □

Steven M. Block is a senior scientist at the Rowland Institute for Science, 100 Cambridge Parkway, Cambridge, Massachusetts 02142, USA and a lecturer in the Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138, USA. S. M. B. thanks Christoph Schmidt for comments on the manuscript. For more information on the LaserTweezers instrument from Cell Robotics, Inc., fill in reader service number 100.

1. Ashkin, A., Dziedzic, J. M., Bjorkholm, J. E. & Chu, S. *Optics Lett.* **11**, 288-290 (1986).
2. Ashkin, A. & Dziedzic, J. M. *Science* **235**, 1517-1520 (1987).
3. Ashkin, A., Dziedzic, J. M. & Yamane, T. *Nature* **330**, 769-771 (1987).
4. Barton, J. P., Alexander, D. R. & Schaub, S. A. *J. appl. Phys.* **64**, 1632-1639 (1988).
5. Barton, J. P. & Alexander, D. R. *J. appl. Phys.* **66**,

- 2800-2802 (1989).
6. Barton, J. P., Alexander, D. R. & Schaub, S. A. *J. appl. Phys.* **66**, 4594-4602 (1989).
7. Wright, W. et al. *IEEE J. quant. Electron.* **26**, 2148-2157 (1990).
8. Bakker Schut, T. C. et al. *Cytometry* **12**, 479-485 (1991).
9. Ashkin, A. *Biophys. J.* **61**, 569-582 (1992).
10. Visscher, K. & Brakenhoff, G. J. *Optik* **89**, 174-180 (1992).
11. Visscher, K. & Brakenhoff, G. J. *Optik* **90**, 57-60 (1992).
12. Burns, M. M., Fournier, J. M. & Golovchenko, J. A. *Science* **249**, 749-754 (1990).
13. Sato, S. et al. *Optics Lett.* **16**, 282-284 (1991).
14. Ashkin, A. & Dziedzic, J. M. *Ber. Bunsenges. phys. Chem.* **93**, 254-260 (1989).
15. Block, S. M. in *Noninvasive Techniques in Cell Biology* Vol. 9, Ch. 15 (eds Foskett, J. K. & Grinstein, S.) 375-402 (Wiley-Liss, New York, 1990).
16. Berns, M. W., Wright, W. H. & Steubing, R. W. *Int. Rev. Cytol.* **129**, 1-44 (1991).
17. Kuo, S. C. & Sheetz, M. P. *Trends Cell Biol.* **2**, 116-118 (1992).
18. Weber, G. & Greulich, K. O. *Int. Rev. Cytol.* **133**, 1-41 (1992).
19. Ashkin, A. & Dziedzic, J. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7914-7918 (1989).
20. Block, S. M., Blair, D. F. & Berg, H. C. *Nature* **338**, 514-518 (1989).
21. Block, S. M., Blair, D. F. & Berg, H. C. *Cytometry* **12**, 492-496 (1991).
22. Charon, N. W. et al. *J. Bact.* **174**, 832-840 (1992).
23. Berg, H. C. *J. theor. Biol.* **56**, 269-273 (1976).
24. Steubing, R. W. et al. *Cytometry* **12**, 505-510 (1991).
25. Seeger, S. et al. *Cytometry* **12**, 497-504 (1991).
26. Berns, M. W. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4539-4543 (1989).
27. Liang, H. et al. *Expl Cell Res.* **197**, 21-35 (1991).
28. Tadir, Y. et al. *Fert. Steril.* **53**, 944-947 (1990).
29. Berns, M. W. et al. *Expl Cell Res.* **198**, 375-378 (1992).
30. Shepherd, G. M. G., Corey, D. P. & Block, S. M. *Proc. natn. Acad. Sci. U.S.A.* **87**, 8627-8631 (1990).
31. Block, S. M., Goldstein, L. S. B. & Schnapp, B. J. *Nature* **348**, 348-352 (1990).
32. Ashkin, A. et al. *Nature* **348**, 346-348 (1990).
33. Kuo, S. C. & Sheetz, M. P. *Biophys. J.* **57**, 399a (1990).
34. Eddidin, M., Kuo, S. C. & Sheetz, M. P. *Science* **254**, 1379-1382 (1991).
35. Kucik, D. F. et al. *J. Cell Biol.* **114**, 1029-1036 (1991).
36. Chu, S. *Scient. Amer.* **266**, 70-76 (February, 1992).
37. Svoboda, K., Schmidt, C. F., Branton, D. & Block, S. M. *Biophys. J.* **63**, 784-793 (1992).
38. Wayne, D. B., Kuo, S. C., & Sheetz, M. P. *J. Cell Biol.* **115**, 102a (1991).
39. Simon, A. & Libchaber, A. *Phys. Rev. Lett.* **68**, 3375-3378 (1992).
40. Misawa, H. et al. *Appl. Phys. Lett.* **60**, 310-312 (1992).
41. Simmons, R. M. et al. in *Mechanism of Myofibrillar Sliding in Muscle Contraction* (eds Sugii, H. & Pollack, G. H.) (Plenum, New York, 1992).
42. Denk, W. & Webb, W. W. *Appl. Opt.* **29**, 2382-2391 (1990).

Custom made
PEPTIDES

Around the 
\$30.00 /residue
Shipped in 10 days

8 to 20 amino acids
20 µmole scale
HPLC analysis & assured quality
Since 1984

Your Source of Peptides
BIO-SYNTHESIS
1-800-227-0627 Fax 214-429-0442

Reader Service No.52

ADVERTISEMENTS

Oligos Etc. Inc.

DNA \$4.50/base
S-DNA \$8/base
Methyl Phosphonate DNA \$8/base
RNA \$8/base
S-RNA \$12/base
O-Methyl RNA \$15/base
Chimerics, Biotin
Modifiers, Fluorescent Tags etc.
No Set-Up Charges

800-888-2358/fax 800-869-0813
Intl.fax 203-438-3637

Reader Service No.30

Synthetic
GENES
GGG GAA AAC GAA TCC
Gly Glu Asn Glu

TIB
MOLBIOL

for further information
FAX +49 30 7127734

Reader Service No.18

Picture perfect

In the picture this week — a one-step photographic copier, new microplates for the radioisotopic and luminescent analysis of adherent cells and an inverted confocal microscope that provides simultaneous imaging of three fluorochromes.

The OneStep photographic copier from Polaroid is designed to offer researchers a time- and cost-saving method of producing 8.25×11 inch photographic enlargements or reduction of images for analysis, reports and publication (*Reader Service No. 101*).

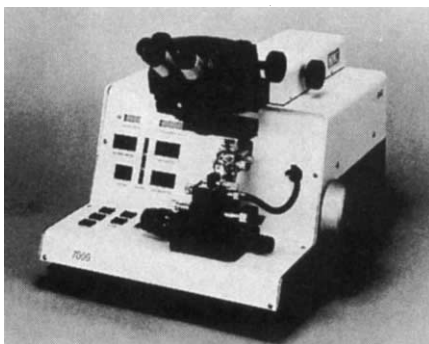


Polaroid's one-step photographic copier.

The photographic copier produces instant print copies, enlargements or reductions and colour overhead transparencies. Polaroid says that it creates 8.25×11 inch colour or black and white enlargements in seconds; full-colour 8.25×11 inch transparencies are ready in two to four minutes. Once the exposed film is peeled, no coating or other processing is required. The OneStep copier also accepts originals as large as 11×17 inches and three-dimensional objects as deep as 0.375 inches. Prints can be reduced or enlarged from 64 to 200 per cent in one per cent increments. The copier measures $32 \times 22 \times 14$ inches and is completely self-contained. It uses no internal chemicals and has a warm-up time of less than 60 s. The film cassettes and negatives used in the copier can be recycled.

Zymed Laboratories has published its new 1992–1993 catalogue, which should be of interest to researchers working in the fields of AIDS research, cancer research, cell biology, flow cytometry, immunohistochemistry and immunology (*Reader Service No. 102*). The catalogue contains an extensive list of procedures and immunochemical products and includes the following new items: directly conjugated $F(ab')_2$ fragments of anti-CD marker antibodies, signal transduction antibodies, liquid DAB, Z-avidin and antibodies for immunohistochemistry. Reagents are available for ELISA, flow cytometry, immunohistochemistry, blotting, immunoprecipitation and antibody purification. A section is also devoted to Zymed's custom, bulk and OEM capabilities.

The MT-7000 Ultra from RMC is a new ultramicrotome that offers ultrathin specimen sectioning for electron microscopy (*Reader Service No. 103*). The stereo microscope is a fully adjustable 'scan and tilt' instrument with an integral eight-way lighting system. The specimen holder also has built-in transillumination, which mounts on either the arc segment mount or trimming post. Other design features include a dual-frame vibration isolator, thick or thin sectioning capabilities up to $10 \mu\text{m}$, a power-driven cutting stroke and microprocessor control. The microprocessor control offers



Sliced and diced with the MT-7000.

a digital display of section count from 0–9,999 with reset and digital display of total advance from 0–999,999 nm with reset. The cutting speed can be varied from $0-49.9 \text{ mm s}^{-1}$ in 0.1 mm s^{-1} increments, and a return speed that is variable. Automatic advance is $200 \mu\text{m}$ with a reset time of less than 30 s. The MT-7000 also features a built-in service routine where autodiagnostic routines are built into the software.

As part of a collaborative effort, Packard and Polyfiltronics have developed a new microplate called ViewPlate that is designed for the radioisotopic and luminescent analysis of adherent cells (*Reader Service No. 104*). ViewPlates are opaque plates with transparent bottoms that permit both microscopic analysis of cell growth and accurate quantitation of adherent cell assays by liquid scintillation or luminescence measurements. An ultrasonic welding process connects the optically transparent bottom plate to a bottomless 96-well black or white microplate frame. Black plates are used to prevent optical crosstalk with luminescent assays and the white plates are used to optimize counting efficiencies and eliminate optical crosstalk with liquid scintillation samples. Both assays can then be analysed in TopCount, Packard's multi-detector microplate scintillation and lumi-

nescence counter. This system uses a single PMT, time-resolved light detection technology to count luminescent, beta- and gamma-labelled samples in the same instrument. Packard says that using ViewPlate with TopCount allows cell growth, assay and analysis all to be performed in the same plate. The plates are treated for cell growth, and a simple washing step is all that is required before analysis with TopCount. The need for time-consuming sample preparation steps, such as solubilization, transfer or harvesting and dispensing in vials or tubes, is eliminated.

■ Image processing/analysis

Jeol has introduced the WINSTOR range of PC-based systems for high-speed, high-resolution processing of microscope images (*Reader Service No. 105*). The company says that WINSTOR not only accepts and displays high-definition images of up to $1,024 \times 1,024$, but can also manipulate and analyse them rapidly — many processes taking less than a second, Jeol says. The system can be used with slowscan and TV-rate signals/spectra, including most



High-resolution PC image processor.

scanning electron microscopes (SEMs), transmission electron microscopes (TEMs) fitted with TV-rate or slowscan TV cameras, variable scan rate imaging scanning tunnelling microscopes (STMs), suitably equipped light microscopes and other scanning sources. Video signals may be displayed 'live' and captured by 'grabbing', averaging or integrating. SEM images are captured via a slowscan generator, which can collect images up to $4,096 \times 4,096$. WINSTOR can also be applied to techniques such as confocal microscopy and STM, which do not produce 'live' images but convert data directly into pictures for easier interpretation. Hard copy can be generated by video or laser printers without leaving the Windows environment. SEM and TEMSCAN-based systems can replay

an image through the microscope's ultra-high resolution record tube to produce high quality negatives. By converting images to TIF format, the microscopist can incorporate them into reports produced by desktop publishing. WINSTOR features floppy and hard disk drives for temporary storage and a built-in WORM-type optical disk for permanent archiving.

GelImage is a new **gel evaluation system** from Pharmacia that consists of a desktop scanner, an evaluation software package and laser printer (*Reader Service No. 106*). The system is designed for the simple and fast documentation and digital evaluation of stained electrophoresis gels and other



Pharmacia's gel evaluation system.

similar samples. Scanned digital images can be exported to a variety of desktop publishing programs for publication or presentation purposes. The system provides a variety of electrophoretic analysis options, including automatic spot identification of two-dimensional gels, three-dimensional representation of the scanned image, analysis of one-dimensional gels, with calculation of molecular weights, isoelectric points and peak area, and manual editing of peaks.

■ TEM

Researchers in medicine, biology and pathology may be interested to hear about a novel feature of Hitachi's H-7100 **transmission electron microscope**. The Low Mag mode is a CPU-controlled function that provides high resolution magnification in ten steps within a range of $\times 50$ to $\times 1,000$, and allows for correlation of the macroscopic image from an optical microscope with the microscopic information from an electron microscope (*Reader Service No. 107*). Most significantly, Hitachi says that the Low Mag mode on the H-7100 allows for wide-field, high-contrast observation of low magnification images with no image distortion. Thus, a low magnification micrograph can be taken of the specimen, which can then be enlarged to give a high-resolution image of a large area. This technique is designed to provide a more convenient and error-free means of imaging a large area of specimen than the old method of piecing together several high magnification images (montaging). Other features include an automatic pre-irradiation system that allows preliminary

specimen treatment for samples that are vulnerable to beam damage, a low-dose facility that allows samples to be observed for longer, and a high-contrast electron column that absorbs scattered electrons and prevents image distortion. The specimen stage is computer-controlled and allows operating features such as autotracking, autoresetting and automontaging, with the specimen stage movements being linked to magnification steps via the computer. An RS232 interface also allows for external computer control of all lens functions and stage movements.

The latest **cryotransfer system** from Oxford Instruments is now available for a wide range of transmission electron microscopes (TEMs) (*Reader Service No. 108*). The CT3500 Crotrans system consists of a high-resolution, low-mass nitrogen cooling holder (0.34-nm resolution, even at high-tilt angles) fitted with Oxford Instruments' 'double shield' technology to avoid contamination of both the specimen and holder during transfer. Biological cell suspensions and frozen sections can be observed in an unfixed vitrified state and cryo-TEM is also a useful complementary technique to X-ray microanalysis. The company says that users have reported that the CT3500 stabilizes very quickly. The company says that this, coupled with no 'waiting time' needed for decontamination in the TEM, means that researchers can reliably and quickly perform an uninterrupted succession of cryotransfers.

■ Confocal microscopy

Carl Zeiss has introduced its new highly modular, user-configured **confocal system** for inverted microscope applications (*Reader Service No. 109*). The LSM 410 inverted confocal microscope allows users



Zeiss' LSM 410 for the simultaneous imaging of three fluorochromes.

to record and display three different fluorescence signals simultaneously with full resolution. The system includes a transmitted-light laser-scan detector and can accommodate up to three separate fluorescence detectors and three lasers. The LSM 410 inverted microscope uses the new Axiovert 100 series inverted research microscopes from Carl Zeiss. The large

unobstructed stage area is suitable for specimen handling with micromanipulators and microinjection systems. The Axiovert 100, 135 and 135M inverted microscopes also offer full TV and photodocumentation capabilities in addition to a multiple-filter slider for conventional epifluorescence experiments. The fluorescence detectors feature individual pinholes that can be continuously varied in size under digital control. Lasers with excitation ranges from 365 nm to 633 nm are available, covering the full spectrum from near-UV to near-infrared wavelengths. Users can switch between different excitation wavelengths without realigning lasers. The system's host computer provides the full capabilities of an IBM-compatible 486 processor, including the DOS 5.0 operating system with Windows 3.1 graphic interface, and a variety of memory upgrades. Software programs such as three-dimensional reconstruction and time-varying evaluation are also available.

An air-cooled Argon/Krypton ion laser is now available as an option for use with the **INSIGHT laser scanning confocal microscope** from Meridian Instruments (*Reader Service No. 110*). The combination of the Ar/Kr laser's multiple lines and INSIGHT's bilateral scanning feature means that researchers can now capture dual-labelled confocal data at video rate with no fluorochrome crosstalk. The Ar/Kr laser has three wavelengths, 488 nm (blue), 568 nm (yellow) and 647 nm (red), which offers researchers the opportunity of using single, dual or triple excitation in their confocal work.

■ Light microscopy

Olympus has launched a new research **zoom stereo microscope**, the SZH-10, which is aimed at high-technology and life science users (*Reader Service No. 111*). The company says that a powerful new $\times 10$ zoom with releasable clickstops for preset magnification is the key to the instrument's flexibility. Also developed specifically for the SZH-10 are the new distortion-free Apochromatic $\times 1$ objectives for sharp edge-to-edge definition. Coaxial fine and coarse focusing and a new counterbalance mechanism improve the microscope's ergonomics. In common with all Olympus system microscopes, the SZH-10 has a highly modular design, allowing the user to specify a virtually made-to-measure instrument. The SZH-10 is compatible with photomicrographic and TV systems.

Nikon has introduced a new accessory that provides most Nikon microscopes with the ability to **zoom continuously over a range from $\times 0.8$ to $\times 2$** (*Reader Service No. 112*). Optizoom allows users to change magnification without changing eyepieces or objectives and overcomes the limitations of fixed, discrete-step objective lens magnification. The Optizoom module is placed

ADVERTISEMENTS

Highest Quality Oligonucleotides

Synthesised, purified and shipped in 3 days

£2.75 per base

(All inclusive)

0.2 µM scale (approx. 500 µg)

- Antisense (completely or partially sulphurised)
- Extensive range of labelling and modifications
- Full design and analysis facilities
- Gene cassettes, bulk order and contract discounts

Molecular Medicine Unit

King's College School of Medicine & Dentistry

Tel. 071 - 326 3126 FAX 071 - 733 3877

Reader Service No.56

WHY PAY MORE?

MINI GEL ELECTROPHORESIS for DNA, RNA & NUCLEOPROTEINS

Model 100 for 92 x 100mm gel with 100ml buffer
or

Model 101 for 80 x 100mm gel with 250ml buffer Gel box with UV-transparent gel support, lid-mounted cables & platinum electrodes and 6, 8, 12 & 16 well-combs from DANAPHOR[®] for safety & SWISS quality. With instructions manual for electrophoresis techniques. Good for up to 2mm thick gels for Southern or Northern blotting.

Price: US \$140. - (freight & insurance extra).

TECTATE S.A.

2, pl. Grand St-Jean,
1003 Lausanne, Switzerland.

for invoice: Fax: 0041-21-311 5783.

Reader Service No.40

PACEMAKERSM

The multiple peptide synthesis and conjugation service

- High purity (up to 95%).
- Quantities for a range of uses.
- Single or multiple peptides.
- HPLC trace with each peptide.
- Terminal modification at no extra charge.
- Conjugation to carrier proteins.

from **£80 per peptide**

AFFINITY Research Products Ltd.
GPT Business Park, Technology Drive,
Nottingham, NG9 2ND, UK.

Tel: +44/0 602 436100; Fax: +44/0 602 436300

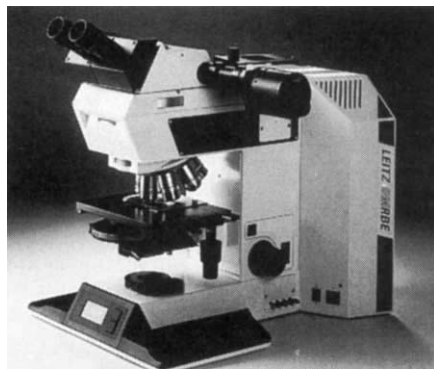
Reader Service No.23

Correction

In the product review article (*Nature* 359, 557-558; 8 October 1992), the name of Gary Gilliland was misspelt.

between the microscope stand and the viewing head to provide an erect, magnified image for visual, photographic and video applications. A built-in reticule holder accommodates two reticules, and the reticules zoom in concert with the Optizoom so that recalibration of the reticule is not required.

The Leitz DM R system from Leica is not one microscope but a total **microscopy system** consisting of nine separate stands, newly computed optics and a choice of electronic focusing and electronic parfocality (*Reader Service No. 113*). Some of



Leica has microscopes for all occasions.

the features of the Leitz DM R system include: new Delta optics; a capacity to integrate both incident and transmitted light contrasting techniques (with 57 possible combinations); electronic focusing; electronic parfocality (an electronic parfocality of 0.1 µm is automatically established when an objective is changed); semi-automatic focusing, which allows stages to be lowered at the press of a button to an individually set end position and back to a pre-set focal plane for each objective; a choice of documentation from simple 35 mm or Polaroid cameras to the new DM RD photosystem with three positions for photography and CCTV plus processor-controlled light metering using a 49-field measuring system; and an optional PC interface for data logging and/or motorized stage control and stage coordinate positioning. Of particular interest to the life scientist will be the new Delta optics comprising about one hundred new objectives in the form of Plan Achromats, Plan Fluotars and Plan Apochromats. These new objectives possess high light transmission characteristics and are suited to fluorescence applications. The condenser system of the DM R is suitable for objectives from $\times 1.6$ to $\times 100$ and maintains automatic Koehler illumination throughout the range, without the need for diaphragm adjustments.

■ Cameras

The Bio Image low-light CCD camera from Millipore allows researchers to acquire high-resolution images ($1,024 \times 1,024$ pixels, noninterlaced) of double-stranded DNA patterns stained with fluorescence

(for example, ethidium bromide) before computer-assisted analysis (*Reader Service No. 114*). Traditionally, images of nonradioactive, double-stranded DNA have been acquired for image analysis by photographing the gel and scanning the photograph. Millipore says that this process can decrease resolution and, ultimately, results in less accurate image analysis. The Bio Image low-light camera captures fluorescent images directly using the Kodak Megapixel imaging chip with MPP circuitry for low dark noise. The images are sharp and crisp, Millipore says, resulting in greater resolution and more precise image analysis. The camera can also be used to acquire images of standard samples (that is, stained gels and autoradiograms) by removing a filter.

The Megaplus camera Model 1.4 from Kodak is designed to capture the high-resolution digital images that are important in, for example, many areas of medical analysis, microscopy and noncontact measurement (*Reader Service No. 115*).



For the full picture see Kodak.

The Megaplus camera captures a high-resolution image, which is digitized by the camera system itself. Once digitized, the image is held on a frame grabber board and is ready for analysis using the computer's software, storage, transmission to another location, and so on. The Megaplus camera offers a sensitivity of up to 256 levels of grey in the captured image, which is particularly important in, for instance, X-ray analysis. Images captured for analysis can later be stored on optical disk, magnetic media or photo CD. Once stored, the images then form a compact archive of high resolution visual data that is accessible by any networked computer terminal. When combined with an optical filter system, the camera can also capture full-colour images by taking three scans (red, green and blue). The camera is compatible with IBM PC, Sun Microsystems, Macintosh and IBM RS6000 computing systems. □

These notes are compiled by Diane Gershon with information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.